Original Paper

MicroRNA-449a Inhibition Protects H9C2 Cells Against Hypoxia/Reoxygenation Induced Injury by Targeting the Notch Signaling Pathway

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Key Words

Apoptosis • Hypoxia/reoxygenation injury • microRNA • Myocardial ischemia-reperfusion injury • Notch

Abstract

Background/Aims: The present study aimed to detect the expression of miR-449a and investigate the effect of miR-449a on cell injury in cardiomyocytes subjected to hypoxia/reoxygenation (H/R) and its underlying mechanisms. **Methods:** The expression of miR-449a was determined using reverse transcription-polymerase chain reaction in both neonatal rat ventricular myocytes and H9C2 cells. For gain-of-function and loss-of-function studies, H9C2 cells were transfected with either miR-449a mimics or miR-449a inhibitor. The target gene of miR-449a was confirmed by a dual-luciferase reporter assay. Apoptosis was analyzed by both flow cytometry using Annexin V and propidium iodide and transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL). Necrosis was confirmed by the detection of lactate dehydrogenase release. The cell viability was measured using the methylthiotetrazole method. The protein levels of Notch-1, Notch-1 intracellular domain, hairy and enhancer of split-1 (Hes-1), and apoptosis-related genes were measured by Western blot analysis. **Results:** miR-449a was significantly upregulated in both neonatal rat ventricular myocytes and H9C2 cells subjected to H/R. However, H/R-induced cell apoptosis and necrosis were markedly reduced by miR-449a inhibition. By targeting Notch-1, miR-449a regulated the Notch-1/Hes-1 signaling pathway. The blockade of the Notch signaling pathway partly abolished the protective effect of miR-449a suppression against H/R injury, whereas the overexpression

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of Notch-1 intracellular domain partly reversed the effect of miR-449a overexpression on H/R-induced cell injury. **Conclusions:** The present study suggested that miR-449a inhibition protected H9C2 cells against H/R-induced cell injury by targeting the Notch-1 signaling pathway, providing a novel insight into the molecular basis of myocardial ischemia-reperfusion injury and a potential therapeutic target.

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Introduction

Acute myocardial infarction (AMI) is the leading cause of death in modern societies worldwide [1]. After the onset of AMI, timely reperfusion by either thrombolytic therapy or primary percutaneous coronary intervention is the most effective strategy to reduce the infarct size [1]. However, restoration of blood to the ischemic myocardium may induce ischemia-reperfusion (I/R) injury and reduce the benefit from reperfusion [1, 2]. No effective strategy to prevent myocardial I/R injury is available to date in clinical practice. Therefore, a better understanding of the molecular mechanisms underlying I/R injury may provide novel insights into the development of effective treatment for coronary heart disease (CHD).

Recent evidence suggests that Notch-1, a major receptor of the Notch signaling pathway, is involved in various hypoxic conditions including myocardial I/R injury [3, 4, 5]. On activation by ligands usually located on the surface of neighboring cells, the Notch receptor undergoes intramembrane proteolysis and releases an intracellular domain (NICD) resulting in signal transduction into the nucleus [4, 6]. These events resulted in the formation of a transcriptional activator complex and induction of the transcription of Notch downstream target genes such as hairy and enhancer of split-1 (Hes-1) [7].

MicroRNAs (miRNAs) are small non-coding RNAs that specifically bind to target mRNAs and thus downregulate gene expression by degradation or translational repression [8, 9]. Recently, these molecules have been implicated in several processes of direct relevance to the heart, including cardiac development, hypertrophy, heart failure, and angiogenesis [10-13]. The altered expression patterns of several miRNAs in myocardium have been confirmed in the setting of ischemia, raising the possibility that miRNAs might affect cardiac function after AMI [14]. Moreover, a number of recent studies demonstrated that efficient inhibition of some miRNAs contributed to the improved recovery of heart function after I/R injury [10, 14-16]. Therefore, modulation of miRNAs to treat myocardial I/R injury is a field of great interest. Better characterization of microRNAs in the heart at baseline and in response to various pathological stimuli is of biological and clinical importance.

This study investigated the role of miR-449a in regulating hypoxia/reoxygenation (H/R) injury, a major *in vitro* model of myocardial I/R injury. Through gain-of-function and loss-of-function experiments, this study explored the mechanism of miR-449a in H/R-induced cell injury and further demonstrated a direct targeting relationship between miR-449a and Notch1 by which miR-449a regulated the Notch signaling pathway. It suggested an inhibitory role for miR-449a in H/R-induced cell injury, indicating a potential target for preventing myocardial I/R injury.

Materials and Methods

Animals

Neonatal Sprague-Dawley rats (1- to 2-day-old) were provided by the Anhui Medical University Laboratory Animal Center (SCXK 2011-002). All animals used in the present study received ethical and humane care. Experimental procedures were conducted in compliance with the Guideline for the Care and Use of Laboratory Animals (Eighth Edition. Washington, DC: The National Academies Press. <https://doi.org/10.17226/12910>) and approved by the Bioethics Committee of Anhui Medical University.

Isolation of neonatal rat ventricular myocytes

Neonatal rat ventricular myocytes (NRVMs) were isolated from the whole heart of Sprague-Dawley rats using a modified protocol. Briefly, the hearts were rinsed with D-Hank's solution to remove blood and then digested with an enzymatic solution containing 0.25% trypsin for 10 min at 37°C. The digestion was terminated by adding Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) at 37°C for 5 min. Subsequently, the hearts were digested with 0.1% type II collagenase at 37°C for 5 min several times until no tissue was visible. Then, the cells were washed and resuspended in DMEM supplemented with 10% FBS and antibiotics. The differential adhesion method was used to remove myocardial fibroblasts, and bromodeoxyuridine (BrdU, 1mM, Sigma, USA) was added into DMEM for the first 24 h to inhibit the growth of myocardial fibroblasts.

Establishment of in vitro H/R model

Rat heart tissue-derived H9C2 cells and NRVM were seeded at a constant density of 1 × 10⁴/well in DMEM containing 10% FBS and antibiotics. The cells were washed with phosphate buffered saline (PBS) and rendered quiescent in serum-free DMEM for 24 h prior to experiments. Both NRVM and H9C2 cells were incubated in a hypoxic chamber supplied with a mixed gas containing 95% N₂ and 5% CO₂ at 37°C for 6 h and then moved to the incubator with 95% air and 5% CO₂ at 37°C for 24 h. Hypoxic medium was replaced by fresh medium upon initiation of reoxygenation. The control plate was kept in the incubator with 95% O₂/5% CO₂ at 37°C. The cells were harvested 12 h post-reoxygenation for analysis.

Lactate dehydrogenase release assay

To determine cell necrosis induced by the H/R injury, the release of lactate dehydrogenase (LDH) in the supernatant was detected using a commercial LDH kit (Takara, China) according to the manufacturer's protocol. Briefly, following the procedure of H/R, culture medium was collected and the adherent cells were lysed according to the manufacturer's protocol. LDH activity was measured using a microplate reader at 490 nm (Biotek Corporation, VT, USA). LDH release was expressed as the ratio of LDH activity in medium to total LDH activity in medium plus lysate and the results were expressed as a percentage.

Assessment of apoptosis in cultured H9C2 cells and NRVM

Apoptosis was measured using flow cytometry and TUNEL staining. For flow cytometry, the cells were harvested and stained with Annexin V fluorescein isothiocyanate and propidium iodide (PI, Thermo Fisher Scientific, Shanghai, China) for 20 min at room temperature. The cells were then washed twice with PBS, and the fluorescence was analyzed with the CellQuest (BD Biosciences, NJ, USA) software. For TUNEL assay, the cells were fixed in 4% paraformaldehyde buffered formalin solution for 30 min. Then, the labeling protocol was used according to the manufacturer's instruction. Apoptotic cells were observed using a fluorescence microscope and quantified by counting at least 100 cells from 4 randomly selected fields in each group.

Cell transfection

MicroRNA mimics, miR-449a inhibitor, and corresponding scrambled control were purchased from Santa Cruz Biotech (Santa Cruz, CA, USA) and transfected into H9C2 cells using Lipofectamine 2000 (Invitrogen, CA, USA) at a final concentration of 50nM according to the manufacturer's protocol. Small interfering RNA (siRNA) targeting Notch-1 (Santa Cruz, TX, USA) was transfected into cells using Lipofectamine 2000 (Invitrogen, CA, USA). The transfection efficacy was determined using reverse transcription-polymerase chain reaction (RT-PCR) or Western blot analysis.

miR-449a mimics: sense: 5'-UGGCAGUGUAUUGUAGCUGGU-3';

antisense: 5'-CAGCUAACAUAACACUGCCA-3'

miR-449a inhibitor: 5'-ACCAGCUAACAUAACACUGCCA-3'

Notch-1 siRNA: sense: 5'-UCGCAUUGACCAUUAACUGGUGG-3';

antisense: 5'-CCACCAGUUUGAAUGGUCAAU-3'

Assessment of cell viability in cultured H9C2 cells

The cell viability was assessed using the methylthiotetrazole (MTT) assay kit (Beyotime, China) according to the manufacturer's protocol and previously reported protocol [17].

Dual-luciferase reporter assay

Both the wild-type 3'-UTR of Notch-1 (GeneBank accession: NM_001105721.1) containing the miR-449a-binding sites, as predicted by TargetScan, and the mutant 3'-UTR of Notch-1 were cloned into a dual-luciferase vector (Promega, USA). For the dual-luciferase reporter assay, 293T cells were plated and co-transfected with the pmirGLO vector and miR-449a mimics using Lipofectamine 2000. After 48 h of transfection, the cells were harvested, and the relative luciferase activity was assessed using the Dual-Luciferase Reporter Assay System (Promega, WI, USA) according to the manufacturer's protocol. A microplate reader (Biotek Corporation, VT, USA) was adopted to test the fluorescence value of firefly luciferase and Renilla luciferase. Renilla luciferase activities were normalized as control.

Plasmid construction and transfection into H9C2 cells

Rat N1ICD cDNA was generated by PCR using the following primers. Forward: 5'-GAGGATCCCCGGGTACCGGTGCCACCATGGTGTCTGTCCGCAAG-3'; reverse: 5'-TCATCCTTGCTAGTCGCTAGCCTTAAATGCCTCTGGAATG-3'. The amplified fragment was cloned into the expression vector pcDNA3.1-Myc-His plasmid (Invitrogen, CA, USA) to increase the expression of Notch-1. Lipofectamine 2000 reagent was used to transfect the H9C2 cells with a pcDNA3.1-N1ICD plasmid according to the manufacturer's protocol.

Real-time quantitative PCR

Total RNA was isolated from the cultured cells using Trizol (Sigma) according to the manufacturer's protocol. Real-time PCR amplification was performed on an Applied Biosystems AB7500 Real-Time PCR system (Applied Biosystems, CA, USA) using SYBR Green PCR Master Mix (Applied Biosystems). The relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method, normalized to the internal control gene β -actin, and then compared with the control group.

The primers used were as follows:

Notch-1 (NM_001105721.1): forward: 5'-ATGACCTCCAGTCTCAAC-3';

reverse: 5'-GTCCAGCCATTGACACACAC-3';

Hes-1 (NM_024360.3): forward: 5'-CAACCTCCGACAAAC-3';

reverse: 5'-CGGAGGTGCTTCACTGTCA-3';

β -actin: forward: 5'-GTGGGGCGTCAAGGAG-3';

reverse: 5'-CTTCCTTAATGTCCTTCGATTTC-3'.

For miRNA analysis, total RNA was isolated using an miRNeasy Mini Kit (Qiagen, Dusseldorf, Germany) and reverse transcribed into cDNA using an miScript Reverse Transcription Kit according to the manufacturer's protocols. The cDNA was synthesized from 400 ng of total RNA using SuperScript III First-Strand Synthesis SuperMix (Life Technologies, Shanghai, China) with random hexamers according to the manufacturer's protocol. The expression of miRNA was assessed by TaqMan microRNA assay (miR-449a, ID 001030) and the expression of miRNA was normalized to that of RNU48 (ID 001006).

Western blot analysis

The cells were lysed on ice for 30 min, and the lysates were clarified by centrifugation at 4°C for 20 min at 12,000 rpm. Then, 20 μ g of total protein was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Millipore, MA, USA). The membranes were blocked for 1 h at 37°C with 5% nonfat dry milk and subsequently incubated with primary antibodies overnight at 4°C according to a previous protocol [17]. The primary antibodies against Notch-1, Notch-1 extracellular domain (Notch-1 ICD), Hes-1, cleaved caspase-3, caspase-3, and β -actin were purchased from Cell Signaling Technology (Shanghai, China). The primary antibodies against B cell lymphoma/leukemia-2 (Bcl-2) and Bcl-2 associated X protein (Bax) were purchased from Abcam (Shanghai, China). The membrane was washed with TBST and incubated with secondary antibody for 2 h at room temperature. The positive protein bands were developed using a chemiluminescent system, scanned, and quantified by densitometry analysis using an image analyzer Quantity One System (Bio-Rad, VA, USA).

Statistical analyses

All measurements were expressed as mean $\pm$ standard error of the mean. The differences between groups were analyzed using either independent-samples *t* test or one-way analysis of variance followed by Student-Newman-Keuls post hoc analyses for pairwise comparison. A *P* value less than 0.05 was considered to be statistically significant.

Results

Expression of miR-449a increased in H9C2 cells after H/R

The validity of the H/R model was evaluated by flow cytometry using Annexin V/PI double staining (Fig. 1A) and TUNEL assay (Fig. 1D). As illustrated in Fig. 1B, the apoptosis rates in the H/R group measured by flow cytometry were $48.12 \pm 3.03\%$ in NRVMs and $42.81 \pm 2.98\%$ in H9C2 cells, which were dramatically higher than those in the corresponding control group ($5.84 \pm 1.31\%$ and $4.15 \pm 1.22\%$, $P < 0.001$, $n = 6$ in each group). In addition, cell necrosis, as indicated by the release of LDH in the H/R group, also increased compared with the control group (Fig. 1C, $P < 0.001$, $n = 6$ in each group). Furthermore, the apoptosis index assessed by TUNEL in the H/R injury group was $22.64 \pm 2.48\%$ in NRVMs and $20.17 \pm 2.27\%$ in H9C2 cells, which was significantly higher than that in the corresponding control group ($4.82 \pm 1.16\%$ and $4.17 \pm 1.05\%$, Fig. 1E, $P < 0.001$, $n = 6$ in each group).

The levels of miR-449a and Notch-1 ICD/Hes-1 in both NRVMs and H9C2 cells in response to H/R injury were examined to determine the potential role of miR-449a in cardiomyocytes that underwent H/R. The expression of miR-449a significantly increased in both NRVMs (6.36-fold) and H9C2 cells (5.31-fold) after H/R, compared with normoxic control (Fig. 1F, $P < 0.001$, $n = 6$ in each group). As shown in Fig. 1G, the mRNA level of Notch-1 significantly decreased 68% in NRVMs and 59% in H9C2 cells, while the level of Hes-1 decreased

Fig. 1. Detection of apoptosis and expression of miR-449a, Notch-1 ICD, and Hes-1 in both NRVM and H9C2 cells. (A) Representative photographs of flow cytometry using Annexin V/PI double staining in NRVM from the control and H/R groups. (B and C) Apoptosis rate and necrosis significantly increased following H/R in both NRVM and H9C2 cells subjected to H/R ($n = 6$ in each group). (D) Representative photographs of TUNEL assay in NRVM from the control and H/R groups. (E) Apoptosis index significantly increased following H/R in both NRVM and H9C2 cells ($n = 6$ in each group). (F) Expression of miR-449a significantly increased in both NRVM and H9C2 cells

subjected to H/R ($n = 6$ in each group). (G–H) mRNA levels of Notch-1 significantly decreased in both NRVM and H9C2 cells subjected to H/R ($n = 6$ in each group). (I–L) Protein levels of Notch-1 ICD and Hes-1 significantly decreased in both NRVM and H9C2 cells subjected to H/R ($n = 6$ in each group). The results shown are mean $\pm$ standard error of the mean from three independent experiments. $\star P < 0.05$ vs control.

36% in NRVMs and 38% in H9C2 cells after H/R, compared with normoxic control ($P < 0.001$, $n = 6$ in each group). In addition, the protein level of Notch-1 ICD significantly decreased 66% in NRVMs and 60% in H9C2 cells, while that of Hes-1 decreased 55% in NRVMs and 38% in H9C2 cells after H/R, compared with normoxic control (Fig. 1I-1L, $P < 0.001$, $n = 6$ in each group). These results suggested the important role of miR-449a in cells that underwent H/R and the involvement of Notch-1/Hes-1 in the process.

Fig. 2. Effect of miR-449a inhibition on H/R-induced cell injury in H9C2 cells.

(A) Representative photographs of flow cytometry using Annexin V/PI double staining. (B) Apoptosis rate significantly increased following H/R in H9C2 cells. miR-449a inhibition dramatically reduced H/R-induced apoptosis in H9C2 cells, as assessed by flow cytometry ($n = 6$ in each group). (C) LDH release significantly increased following H/R in H9C2 cells. miR-449a inhibition dramatically reduced H/R-induced LDH release in H9C2 cells ($n = 6$ in each group). (D) Cell viability was detected by MTT assay. The result showed no significant difference in cell viability among the control, H/R, miR-449a inhibitor, and scramble control groups. (E) Representative photographs of TUNEL assay in H9C2 cells (control and H/R). (F) Apoptosis index significantly increased in H9C2 cardiomyocytes subjected to H/R. miR-449a inhibition significantly reduced H/R-induced apoptosis in H9C2 cardiomyocytes, as assessed by TUNEL assay. (G-H) The Bcl-2/Bax ratio significantly decreased in the H/R group. miR-449a inhibition resulted in a noticeable increase in the Bcl-2/Bax ratio compared with the H/R group. (I-K) The levels of cleaved caspase-3 protein significantly increased in H9C2 cells that underwent H/R, and this increase was blocked by suppression of miR-449a ($n = 6$ in each group). The results shown are mean $\pm$ standard error of the mean from three independent experiments. $\star P < 0.05$ vs control. $\blacklozenge P < 0.05$ vs H/R group.

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promoted the expression of both mRNA (Fig. 3D) and protein (Fig. 3B and 3E). Overall, these results indicated that Notch1 was a direct gene of miR-449a.

miR-449a inhibition upregulated Notch signaling in the context of H/R

The effect of miR-449a inhibition on Notch signaling was determined to further investigate the underlying molecular mechanisms of miR-449a in regulating H/R-induced apoptosis. The results showed that NICD protein levels were significantly increased by miR-449a inhibition compared with those in the H/R group (2.51-fold, $P < 0.001$, Fig. 3F and 3H). Moreover, the protein levels of downstream target genes of Notch signaling Hes-1 were significantly increased by miR-449a inhibition (3.11-fold, $P < 0.001$, Fig. 3G-3H). In addition, the mRNA levels of Hes-1 also significantly increased by miR-449a inhibition (2.1-fold, $P < 0.001$, Fig. 3I). The results suggested that miR-449a inhibition promoted the activation of Notch signaling.

Knockdown of Notch-1 abolished the protective effects of miR-449a inhibition on apoptosis, necrosis, and the expression of Notch-1/Hes-1 in H9C2 cells

The expression of Notch1 was silenced, together with miR-449a inhibition, to confirm that Notch1 was involved in miR-449a inhibition-induced protective effects against H/R. As predicted, the protective effect of miR-449a inhibition against H/R-induced apoptosis (Fig. 4A-4B) and necrosis (Fig. 4C) was partly reversed by Notch1 silencing. In addition, the elevated expression of Notch1 caused by miR-449a inhibition was partly abolished by

Fig. 5. Blocking of Notch signaling abolished the protective effects of miR-449a inhibition. (A) Representative photographs of flow cytometry using Annexin V/PI double staining in H9C2 cells. (B and C) The protective effects of miR-449a inhibition against H/R-induced apoptosis and LDH release were markedly reversed by pretreatment with Notch-1 signaling blocker DAPT (10mM). (D and E) The elevated expression of NICD caused by miR-449a inhibition was significantly abolished by pretreatment with Notch-1 signaling blocker DAPT (10mM). (F and G) The increased protein levels of miR-449a inhibition-induced Hes-1 also markedly decreased by pretreatment with Notch-1 signaling blocker DAPT (10mM). ★ $P < 0.05$ vs control; ♦ $P < 0.05$ vs H/R group; # $P < 0.05$ vs miR inhibitor group ($n = 6$ in each group). The results shown are mean $\pm$ standard error of the mean from three independent experiments.

Notch1 silencing (Fig. 4D-4E). Similarly, the increased protein levels of NICD and miR-449a inhibition-induced Hes-1 were also decreased by Notch1 silencing (Fig. 4F-4I). Overall, these results suggested that miR-449a inhibition protected cells against H/R-induced apoptosis through promoting the expression of Notch1.

Blocking Notch signaling abolished the protective effects of miR-449a inhibition on apoptosis, necrosis, and the expression of Notch-1/Hes-1 in H9C2 cells

Notch signaling was blocked using DAPT (Sigma-Aldrich, 10 mmol/L), and then the effect on miR-449a inhibition-induced protective effects was detected to further verify that the Notch-1/Hes-1 signaling pathway was involved in miR-449a inhibition-induced protective effects against H/R. The results showed that blocking Notch signaling abolished the protective effects of miR-449a inhibition against H/R-induced apoptosis (Fig. 5A and 5B) and necrosis (Fig. 5C). Furthermore, the results also showed that the increased protein levels of NICD and miR-449a inhibition-induced Hes-1 also markedly decreased by Notch1 blocking (Fig. 5D-5G), implying that miR-449a inhibition protected H9C2 cells against H/R-induced apoptosis through regulating the Notch signaling pathway.

Fig. 6. Overexpression of N1ICD reversed the effect of the overexpression of miR-449a on apoptosis and Notch-1/Hes-1 expression in H9C2 cells. The cells were co-transfected with miR-449a mimic and N1ICD plasmid for 48 h and then subjected to H/R. (A) Representative photographs of flow cytometry using Annexin V/PI double staining in H9C2 cells. (B and C) Overexpression of miR-449a exacerbated H/R-induced apoptosis in H9C2 cells. This effect was significantly abrogated by the overexpression of NICD. (D and E) The decreased expression of NICD caused by the overexpression of miR-449a was partly abolished by the overexpression of NICD. (F and G) The decreased protein levels of Hes-1 induced by the overexpression of miR-449a also markedly decreased by the overexpression of NICD. ★P < 0.05 vs control; ◆P < 0.05 vs H/R group; #P < 0.05 vs miR mimic group (n = 6 in each group). The results shown are mean ± standard error of the mean from three independent experiments.

Overexpression of NICD reversed the effect of the overexpression of miR-449a on apoptosis, necrosis, and the expression of Notch-1/Hes-1 in H9C2 cells

A rescue assay by overexpressing NICD was performed to further verify that miR-449a functioned directly through Notch-1 signaling. The results showed that the overexpression of miR-449a by miR-449a mimic transfection exacerbated H/R-induced injury in H9C2 cells, as evident by flow cytometry and LDH release assay. As expected, this effect was significantly abrogated by the overexpression of NICD (Fig. 6A-6C). In addition, the decreased expression of NICD induced by the overexpression of miR-449a was partially restored by overexpression of NICD (Fig. 6D-6E). Moreover, the expression of Hes1, which decreased by miR-449a overexpression, was also significantly rescued by the overexpression of NICD (Fig. 6F-6G). In summary, these results suggested that miR-449a functioned through Notch-1 signaling in the regulation of H/R-induced injury.

Discussion

The present study found that the expression of miR-449a was markedly upregulated in both NRVMs and H9C2 cells subjected to H/R, along with increased cell apoptosis and necrosis as well as downregulation of the expression of Notch-1 and Hes-1. The study revealed that the suppression of miR-449a attenuated H/R-induced apoptosis and necrosis in H9C2 cells by targeting Notch-1 and inhibiting the Notch signaling pathway, thereby altering the expression of apoptosis-related proteins including Bcl-2, Bax, and caspase-3. These results suggested miR-449a might contribute to the development of H/R injury, representing the therapeutic potential of miR-449a in myocardial I/R injury.

Numerous studies have established that cell death of cardiomyocytes through regulated cell suicide mechanisms contributes critically to the pathogenesis of many cardiovascular diseases including myocardial I/R injury and heart failure [18]. The most important cell death programs in the context of heart disease are apoptosis and necrosis. As a programmed cell death, apoptosis is considered one of the crucial pathological processes in myocardial I/R injury. In contrast, necrosis is usually considered a nonprogrammed cell death that occurs in response to a toxic/ischemic injury [2]. The amount of cell death determines the severity of I/R injury. In addition, as the number of cardiomyocytes decreases, the heart may undergo compensatory cardiac hypertrophy, ventricular remodeling, and, ultimately, heart failure. It is necessary to explore the mechanisms that trigger cell death in cardiomyocytes during I/R injury and the potential prevention strategies for inhibiting myocardial apoptosis and death after I/R injury and the consequent heart failure [19, 20]. Current evidence suggests that miRNAs are vital in regulating myocardial apoptosis during I/R injury. For instance, Rane et al. reported that the expression of miR-199a was dramatically downregulated in cardiac myocytes suffering from hypoxia. Moreover, the overexpression of miR-199a reduced hypoxia-induced apoptosis [21]. Similarly, miR-21 was reported to exert a protective effect on H/R-induced cell apoptosis, which was associated with its targeting of the programmed cell death 4 [10]. A large number of miRNAs, such as miR-29, miR-226, miR-320, and miR-494, were proposed in the process of myocardial I/R injury [15, 16, 22, 23]. These findings implied that the myocardial I/R injury was regulated by various miRNAs that might form a complex regulatory network. Marcet and his colleagues previously demonstrated that the members of miR-34/449 family shared high sequence homology [24, 25]. miR-34 was confirmed to be a tumor suppressor, and its level was relatively low in the cardiovascular system. Similarly, previous studies also suggested that miR-449a was a tumor suppressor in numerous cancer types by targeting different target genes [8, 26, 27]. Moreover, a number of studies recently indicated the downregulation of the expression of miR-449 in various human malignancies, including lung cancer, prostate cancer, and adrenal hyperplasia [28, 29]. miR-34 has been reported to function in cardiovascular disorders by regulating apoptosis, telomere attrition, DNA damage, and inflammatory response [30-33]. However, no study has reported the involvement of miR-449 in the cardiovascular disease.

The present study showed that miR-449a was a novel miRNA that regulated cardiomyocyte death resulting from H/R injury. It found that miR-449a level significantly increased in both NRVM and H9C2 cells induced by H/R injury. The effect of miR-449a inhibition was observed by gain-of-function and loss-of-function studies in H9C2 cells, a cardiac muscle lineage widely used in functional studies of cardiomyocytes *in vitro*. The present study demonstrated that miR-449a inhibition protected H9C2 cells against H/R injury, including both apoptosis and necrosis, by targeting Notch-1. These results suggested that miR-449a might be an important regulative miRNA that responded to H/R conditions in cardiomyocytes and provided therapeutic targets for treating myocardial I/R injury and heart failure.

A number of cellular signaling pathways are speculated to be involved in the process of myocardial I/R injury. Abundant evidence from previous studies has demonstrated that Notch is a class of highly conserved cell-surface receptors [5, 7]. Notch is a transmembrane protein that consists of an extracellular domain and an intracellular domain. In the canonical Notch-1 signaling pathway, the intracellular domain is hydrolyzed twice by the matrix metalloproteinase (MMP)-13 and γ -secretase into the activated Notch-1 intracellular domain (NICD), after the binding of extracellular domain to the ligand. Then, NICD translocates into the nucleus and binds to the transcriptional factor Notch-1/suppressor of hairless/LAG-1 (CSL), to activate the expression of downstream genes [4, 6]. Four isoforms of Notch have been found in mammals: Notch 1–4. Numerous studies have demonstrated that the Notch signaling pathway participated in the regulation of cardiomyocyte proliferation, myocardial trabeculation, and valve formation, as well as the maintenance of adult heart tissue integrity [3, 34, 35]. Particularly, Notch-1 is an important adaptive signaling pathway that participates in I/R injury of various organs, especially the heart [36–39]. In H9C2 cells, the activation of the Notch 1 gene was demonstrated to effectively promote cell proliferation and inhibit apoptosis in ischemic post-conditioning [40]. Consistently, Notch-1 can protect cardiomyocytes against hypoxia or I/R injury [41]. These findings support that Notch activation is a favorable signal in myocardial I/R injury. In the present study, the expression of Notch-ICD dramatically decreased in H/R-exposed NRVM and H9C2 cells. However, suppression of miR-449a by H/R exposure significantly alleviated the Notch-ICD downregulation. This mechanism was crucial in the protective action of miR-449a inhibition on H9C2 cells. In fact, the complete rescue of miR-449a inhibition to increase cell viability by reducing H/R-induced cell injury was significantly hampered by transfection of siRNA targeting Notch-1 and administration of the γ -secretase inhibitor DAPT, which blocked the generation of Notch-1 ICD. On the contrary, both the downregulation of Notch-1 ICD/Hes-1 and enhanced cell injury were partly reversed by the overexpression of Notch-1 ICD. Taken together, these results implied that, in the context of H/R, miR-449a inhibition alleviated cell injury by targeting the Notch-1/Hes-1 signaling pathway.

In summary, the present study found that miR-449a could target and regulate Notch1 in H9C2 cells. It elucidated that the expression of miR-449a was upregulated in both NRVM and H9C2 cells in the case of H/R. The increased expression of miR-449a might contribute to the decreased expression of Notch1, leading to the inactivation of the Notch signaling pathway during H/R injury. In addition, the present study demonstrated that miR-449a inhibition resulted in alleviated cell injury by the targeting Notch-1/Hes-1 signaling pathway. These findings might provide novel insights into the understanding of the molecular basis of myocardial I/R injury and provide clues for future studying pharmacological treatments.

The present study has some limitations. Firstly, cell injury was measured after reoxygenation. Thus, hypoxia induced cell injury might partly account for the results of both flow cytometry and LDH release assay. Nevertheless, the protective effect of miR-449a inhibition was confirmed by subsequent loss- and gain-of-function study. Second, the present findings were obtained from cultured neonatal cardiac muscle cells and H9C2 cells using an *in vitro* cellular model, which may not exactly behave as adult cardiomyocytes of the heart *in vivo*. Therefore, further studies of the effect of miR-449a on myocardial I/R injury and heart failure in an *in vivo* animal model are warranted.

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Disclosure Statement

The authors declare to have no conflict of interests.

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