MicroRNA-590-3p relieves hypoxia/reoxygenation induced cardiomyocytes apoptosis and autophagy by targeting HIF-1α

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Abstract. Autophagy and apoptosis are key factors in myocardial ischemia/reperfusion (I/R) injury. MicroRNAs (miRNAs or miRs) participate in occurrence and development of myocardial I/R injury by regulating autophagy and apoptosis. The purpose of the present study was to investigate the role of miR-590-3p in the regulation of autophagy and apoptosis in hypoxia/reoxygenation (H/R)-treated cardiomyocytes. Following 6 h hypoxia and 6 h reoxygenation in primary rat cardiomyocytes, miR-590-3p was downregulated. Transfection of miR-590-3p mimic inhibited the increased autophagy and apoptosis following H/R treatment. Subsequent experiments demonstrated that miR-590-3p regulated induction of autophagy and apoptosis by targeting hypoxia inducible factor (HIF)-1α. Forced expression of HIF-1α rescued the protective effect of miR-590-3p on H/R-induced cardiomyocytes. In summary, the present study showed that miR-590-3p exhibited a protective effect on H/R-induced cardiomyocyte injury and may be a novel target for the treatment of myocardial ischemia disease.

Introduction

Myocardial ischemia/reperfusion (I/R) injury begins with cardiac ischemia; reperfusion leads to increased cell loss and enlarged infarction area (1). Restoration of blood flow following infarction increases myocardial structural and functional damage, resulting in cardiac dysfunction, myocardial shock and malignant arrhythmia (2,3). Decreasing myocardial I/R injury can lessen infarct size, thereby improving long-term cardiac function. Therefore, it is important to understand the molecular mechanism of I/R injury, find effective targets and prevent myocardial cell loss following I/R injury.

Autophagy and apoptosis are the two types of programmed cell death (4). Inhibiting apoptosis decreases myocardial damage following infarction. The role of autophagy has been studied in many fields, such as cancer and cardiovascular disease (5,6). Autophagy is widespread in myocardial I/R injury (7) and its role may be protective or destructive (8). In the early stage of ischemia, autophagy facilitates cardiomyocyte survival, but with the prolongation of ischemia, the increase of autophagy may cause cell death and lead to heart failure (9). Controlling the autophagy pathway during myocardial I/R may serve a protective or damaging role. It is crucial to selectively block undesirable pathways involved in autophagy without damaging beneficial pathways. For example, exosomes derived from mesenchymal stem cells alleviate myocardial ischemia reperfusion by inducing cardiomyocyte autophagy via AMPK/mTOR and Akt/mTOR pathways (10). However, miR-103a-3p relieves apoptosis and autophagy in hypoxia-induced H9c2 cells by targeting Atg5 (11)

Recent studies (12-14) have found a promising way to relieve or eliminate I/R injury following myocardial infarction by controlling miRNA expression. MicroRNAs (miRNAs or miRs) are a class of small non-coding RNA with 21-23 nucleotides that inhibit gene expression by binding to the 3' untranslated region (UTR) of mRNA (15,16). A large number of miRNAs and mRNAs constitute a complex regulatory network (17). A single miRNA may regulate hundreds of different target mRNAs, while a single mRNA may also be regulated by several miRNAs (18). miRNAs have been reported to be involved in the pathological process of cardiovascular disease, especially in the pathophysiological progress of myocardial apoptosis, arrhythmia, heart failure and cardiac hypertrophy (19,20) but they do not have protein-coding capabilities (21). There is increasing

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evidence (9,15) that miRNAs affect autophagy or apoptosis during myocardial I/R injury. For example, miR-103a-3p serves a protective role in myocardial ischemia by affecting autophagy (11) and miR-496 inhibits cardiomyocyte apoptosis and protects ischemic myocardial tissue (22). miR-590-3p is produced by its precursor mir-590 and its role in cancer has been widely reported (14); miR-590-3p serves an important role in myocardial fibrosis. For example, miR-590-3p inhibits cardiac fibroblast proliferation, differentiation, migration and collagen synthesis by targeting zinc finger E-box binding homeobox 1 (23). In addition, immediate injection of synthetic miR-590-3p lipid formulations on the anterior ventricular wall following myocardial infarction in mice decreases infarct size and promotes recovery of myocardial function (24). To the best of our knowledge, however, the expression of miR-590-3p following cardiac I/R and the role of miR-590-3p in cardiac I/R injury have not been reported.

Materials and methods

Isolation of primary rat cardiomyocytes. A total of 15 Neonatal Sprague-Dawley (SD) rats (1-3 days, sex indeterminable, 5-6 g) were purchased from the Experimental Animal Center of Bengbu Medical College (Bengbu, China; lot no. 20180004002485). All animal procedures complied with the United States National Institutes of Health Guide (25) and were approved by the Animal Ethics Association of Bengbu Medical College (approval no. 075, 2017). Primary cardiomyocytes were isolated under sterile conditions, as previously described (26). Following anesthesia with 4% chloral hydrate, rats were euthanized via cervical dislocation. Death was confirmed by pupil dilation and cessation of heartbeat. The ventricles was removed, washed with cold PBS, cut into 1-mm^3 pieces and digested in 0.1% II type collagenase at 37˚C for 5 min. The digestion process was repeated 4-5 times. In order to isolate fibroblasts, cell suspensions ($1x10^5$/ml)were cultured at 37˚C for 1.5 h, then non-adherent cells were collected and cultured in DMEM F12 containing 10% FBS (both HyClone; Cytiva) in CO_2 at 37˚C. A total of 100 μmol/l 5-bromo-2-deoxyuridine was added to the medium to prevent proliferation of cardiac fibroblasts.

Troponin C (TNNC1) is a specific protein of cardiomyocytes (27). Anti-cardiac TNNC1 antibody (1:300; cat. no. BA4613; Wuhan Boster Biological Technology Ltd.) was used to identify cardiomyocytes by Immunofluorescence. Cell slides were incubated with Anti-cardiac TNNC1 overnight at 4˚C after blocking with 5% bovine serum albumin (BSA; Gibco; Thermo Fisher Scientific, Inc.) for 30 min at 37˚C. FITC-conjugated secondary antibodies (1:200; cat. no. S0008; Affinity Biosciences) were added and incubated in 37˚C for 1h. The nuclei of primary cardiomyocytes were stained with DAPI for 10 min in 37˚C. Fluorescent images were obtained with fluorescence microscope camera (OLYMPUSU-HGLGPS).

Experimental groups and interventions. In order to mimic H/R injury, cells ($1x10^5$/ml) were cultured in a hypoxic incubator with 1% O_2, 94% N_2 and 5% CO_2 at 37˚C for 6 h. Then, cells were reoxygenated (95% air, 5% CO_2) for 6 h. psiCHECK2-HIF-1α plasmid and control psiCHECK2 plasmid were commercially provided by Hunan Fenghui Biotechnology Co., Ltd. Cell transfection was performed using Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) in accordance with the manufacturer's instruction. Primary cardiomyocytes were divided into the following groups: Control (untreated primary cardiomyocytes), H/R (H/R-treated primary cardiomyocytes), miR-negative control (NC) + H/R (transfection with 100 μg of miR-590-3p mimic NC in 37˚C for 6 h, then H/R treatment), mimic + H/R (transfection with 100 μg of miR-590-3p mimic in 37˚C for 6 h, then H/R treatment), mimic + vector + H/R (co-transfection with 100 μg of miR-590-3p mimic and 0.8 μg of control psiCHECK2 plasmid in 37˚C for 6 h, then H/R treatment) and mimic + HIF-1α + H/R (co-transfection with 100 μg of miR-590-3p mimic and 0.8 μg of psiCHECK2-HIF-1α plasmid, then H/R treatment). H/R treatment was performed 24 h after transfection. The sequence of miR-590-3p mimic and mimic NC were: miR-590-3p mimic sense, 5'-UAAUUUUAUGUAUAAGCUAGU-3' and antisense, 5'-UUCUCCGAACGUGUCACGUTT-3' and miR-590-3p mimic NC sense, 5'-UAGCUUAUACAUAAAAUUAUU-3' and antisense, 5'-ACGUGACACGUUCGGAGAATT-3'.

Reverse transcription-quantitative (RT-q)PCR. Total RNA was extracted from primary cardiomyocytes using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Purity and concentration of total RNA were detected using a microplate reader (Biotek™ Epoch™; BioTek Instruments, Inc.). Total RNA (2 μg) was used to synthesize cDNA using an All-in-One™ miRNA RT-qPCR Detection kit (GeneCopoeia, Inc.) at 37˚C for 60 min and 85˚C for 5 min. The primers were as follows: miR-590-3p forward, 5'-CGCTAATTTTATGTATAAGCTAGTAAAA-3' and reverse, 5'-TGGTGTCGTGGAGTCG-3' and U6 forward, 5'-AUAAAUCCCUUUACACCUCTT-3' and reverse, 5'-AAUAAAUCCCUUUACACCUCTT-3'. The miR-590-3p expression level was quantified using 20 μl reaction mixture containing 2.0 cDNA (20 ng/μl), 2.0 each forward reverse primer, 10.0 All-in-One qPCR Mix and 0.4 μl Rox reference dye (from the kit). The thermocycling conditions were as follows: 95˚C for 10 min, then 40 cycles at 95˚C for 10 sec, 60˚C for 20 sec and 72˚C for 10 sec. The relative expression of miR-590-3p was analyzed by $2^{-\Delta\Delta Cq}$ value calculation method (28) and normalized to the internal control, U6. Each experiment was repeated three times.

Detection of cell apoptosis. Annexin V-FITC/PI apoptosis detection kit (Beyotime Institute of Biotechnology) was used to detect cell apoptosis by flow cytometry. Following treatment as aforementioned, cells were collected and resuspended with 1X binding buffer. Cell suspension was adjusted to a concentration of $1x10^6$ cells/ml. Subsequently, 5 μl Annexin V-FITC and 10 μl PI were added and vortexed gently, then incubated at room temperature (20-25˚C) for 15 min in the dark. Cells were analyzed using FACSCalibur™ (Becton, Dickinson and Company). The percentage of cells in each quadrant were

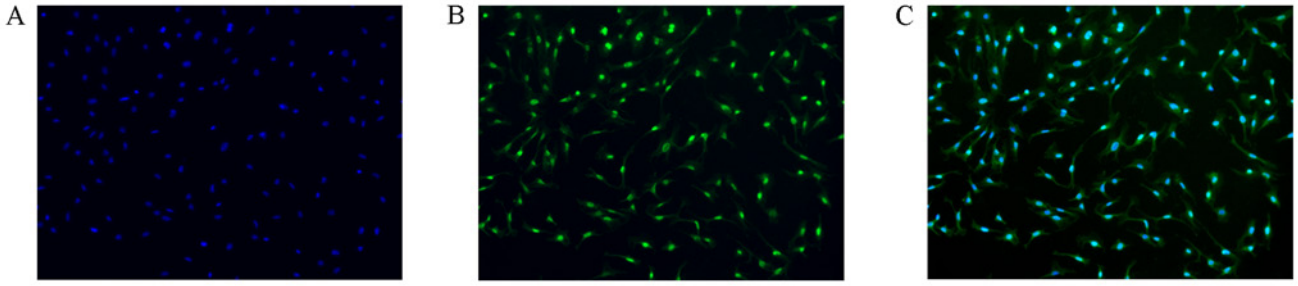

Figure 1. Identification of primary cardiomyocytes by immunofluorescence. (A) DAPI. (B) Troponin C. (C) Merged. Magnification, x100.

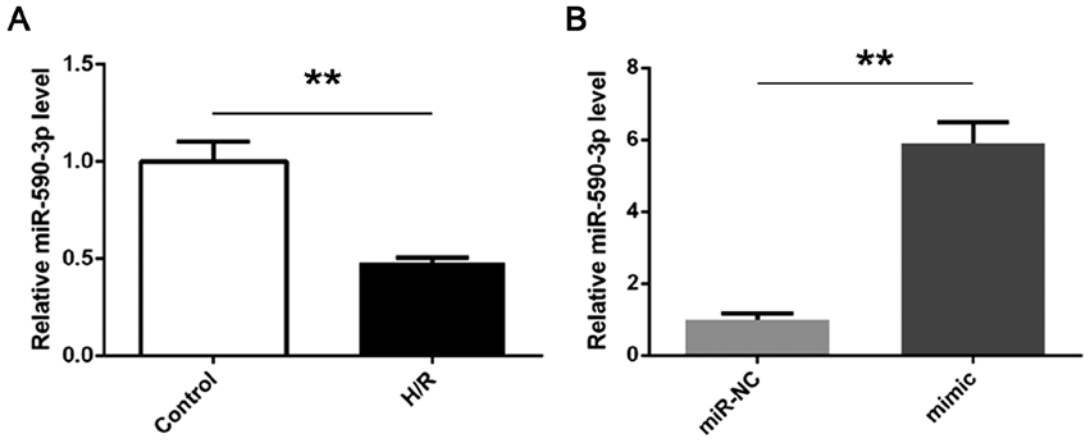

Figure 2. Expression levels of miR-590-3p. Expression levels of miR-590-3p in (A) control and H/R groups and (B) following transfection of mimic. **P<0.01. miR, microRNA; H/R, hypoxia/reoxygenation; NC, negative control.

analyzed using ModFit software (Verity Software House, Inc.). Each experiment was repeated three times.

Western blot analysis. Following treatment as aforementioned, cells were collected and lysed with lysis buffer (Beyotime Institute of Biotechnology) containing 0.5 mM PMSF (Sigma-Aldrich; Merck KGaA). Protein concentration was determined by BCA kit (Beyotime Institute of Biotechnology). Equal amounts of protein (40 μg) was subjected to 10% SDS-PAGE gels and then transferred to a polyvinylidene difluoride membrane. The membranes were blocked with 5% free-fat milk for 2 h at room temperature, and subsequently incubated with the following primary antibodies at 4˚C overnight: Bcl-2 (1:1,000; cat. no. AF6139; Affinity Biosciences), Bax (1:1,000; cat. no. AF0120; Affinity Biosciences), cleaved caspase-3 (1:1,000; cat. no. AF7022; Affinity Biosciences), light chain (LC)3 (1:1,000; cat. no. ab51520; Abcam), p62 (1:1,000; cat. no. ab56416; Abcam), Beclin-1 (1:1,000; cat. no. ab62557; Abcam), HIF-1α (1:1,000 cat. no. ab216842; Abcam) and β-actin (1:3,000, cat. no. AF7018, Affinity Biosciences). Then the membrane was incubated with HRP-conjugated secondary antibody (1:10,000; cat. no. BL003A; Biosharp) for 2 h at room temperature. Proteins were identified by electrochemiluminescence (Beyotime Institute of Biotechnology) and imaged using X-ray film. The intensities of protein were quantified by Bio-Rad Quantity One v4.62 software (Bio-Rad Laboratories, Inc.). Each experiment was repeated three times.

Dual luciferase reporter assay. TargetScan (29) database was used to identify miR-590-3p target genes. Dual-Lumi™ Luciferase Assay kit (Beyotime Institute of Biotechnology) was used for dual luciferase reporter assay according to the manufacturer's instruction. Human embryonic kidney cells (HEK293, $1x10^5$/ml) were cultured in a 24-well plate in CO_2 at 37˚C and then co-transfected with pGL3-HIF-1α-3'UTR/pGL3-HIF-1α mutant 3'UTR plasmid (Promega Corporation) and mimic control/miR-590-3p mimic using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instruction. Following 48 h of incubation, the luciferase activity was measured using dual luciferase reporter assay kit (Beyotime Institute of Biotechnology) according to the manufacturer's protocol. Each experiment was repeated three times.

Statistical analysis. Data are presented as the mean ± SD. Each experiment was repeated three times. Data were analyzed by GraphPad Prism software 7.0 (GraphPad Software, Inc.). Data from two groups were compared by paired Student's t-test; data from multiple groups were compared using one-way analysis of variance followed by Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Immunofluorescence identification of primary cardiomyocytes. Primary cardiomyocytes were observed by fluorescence microscopy using TNNC1 as a specific

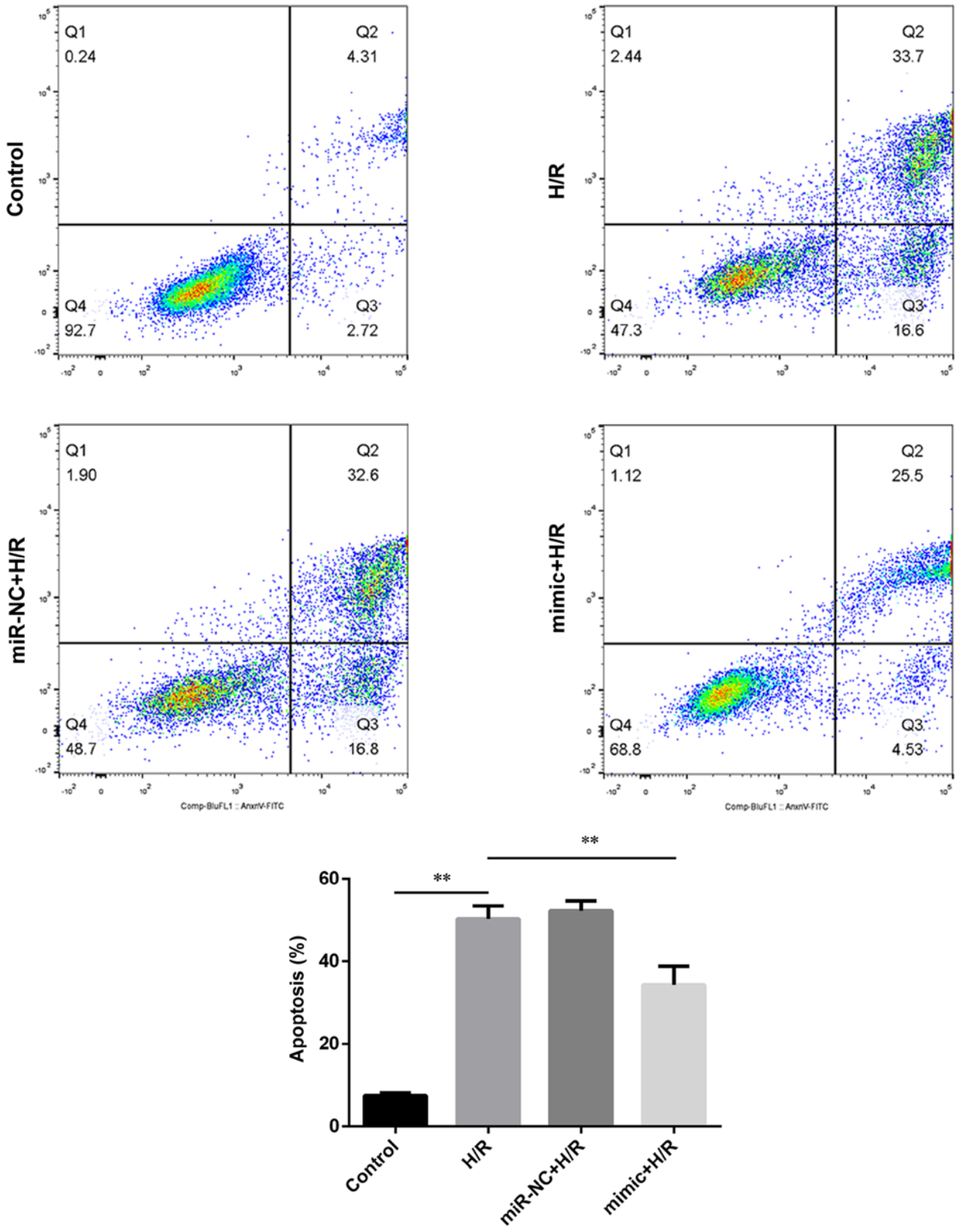

Figure 3. miR-590-3p inhibits apoptosis of H/R-treated cardiomyocytes. Cell apoptosis was measured by flow cytometry. **P<0.01. miR, microRNA; H/R, hypoxia/reoxygenation; NC, negative control.

cardiomyocyte protein. Green fluorescence was located in the cytoplasm of cardiomyocytes; blue fluorescence was located in the nucleus of all cells. The purity of cultured cardiomyocytes was >95% (Fig. 1).

Expression of miR-590-3p decreases in primary cardiomyocytes following H/R treatment. In order to determine expression of miR-590-3p, RT-qPCR was performed in H/R and control groups. The results indicated that compared with the control group, the

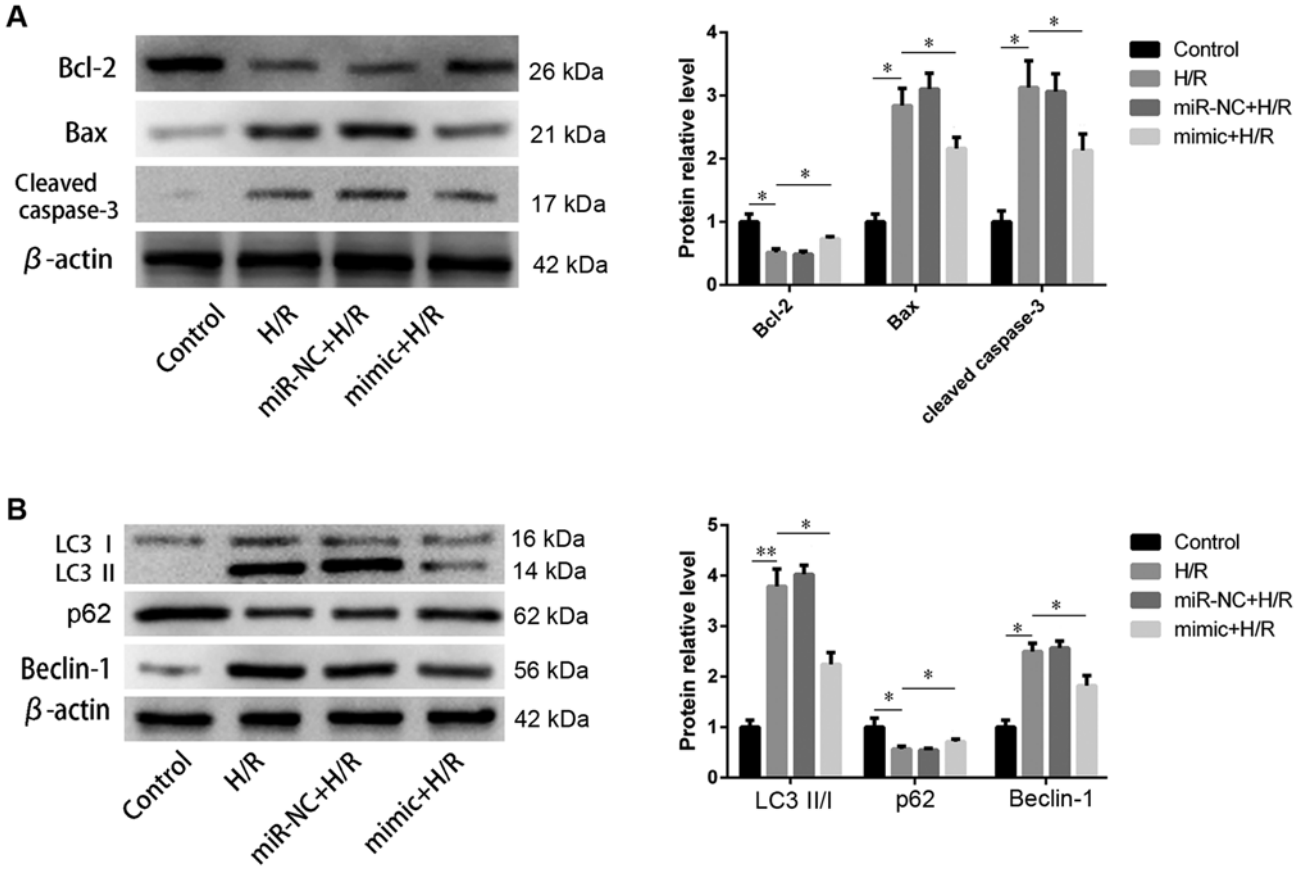

Figure 4. miR-590-3p inhibits autophagy- and apoptosis-associated protein expression levels in H/R-treated cardiomyocytes. Expression of (A) apoptosis- and (B) autophagy-associated proteins. *P<0.05 and **P<0.01. miR, microRNA; H/R, hypoxia/reoxygenation; LC, light chain; NC, negative control.

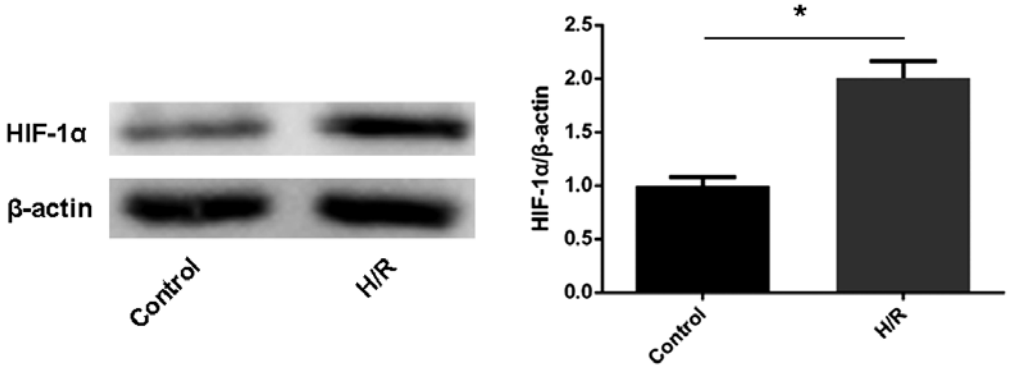

Figure 5. HIF-1α is upregulated following H/R treatment. *P<0.05. HIF-1α, hypoxia inducible factor-1α; H/R, hypoxia/reoxygenation.

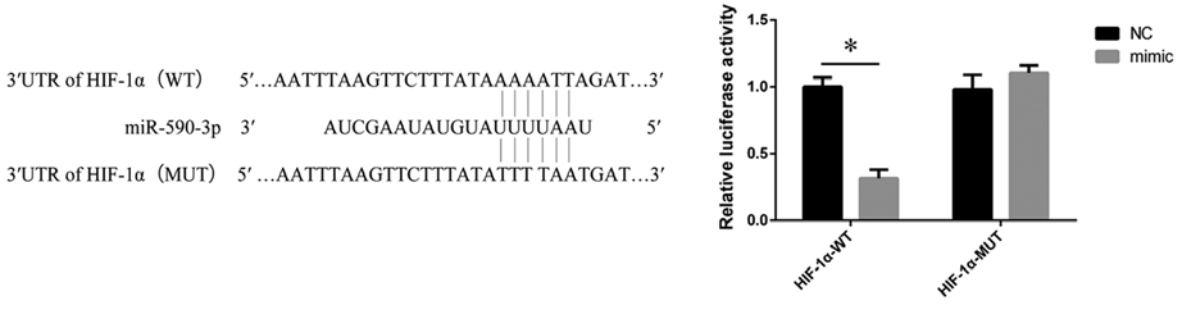

Figure 6. HIF-1α is a target gene of miR-590-3p. Bioinformatics analysis indicated that HIF-1α contains a binding site of miR-590-3p. Luciferase activity was measured following co-transfection with miR-590-3p mimic or NC and WT or MUT HIF-1α 3'UTR. *P<0.05. HIF-1α, hypoxia inducible factor-1α; miR, microRNA; NC, negative control; MUT, mutant; UTR, untranslated region; WT, wild-type.

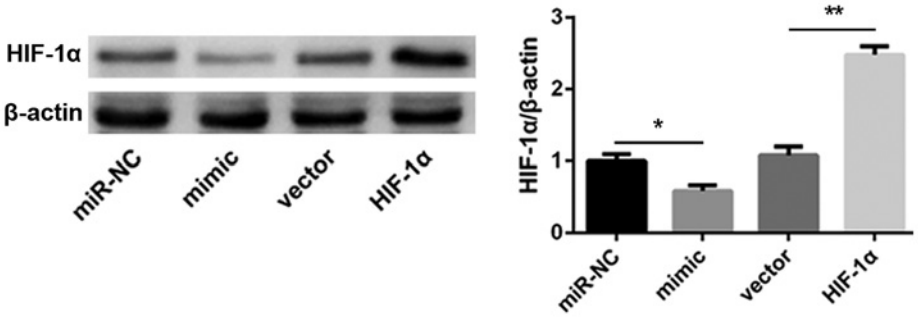

Figure 7. miR-590-3p suppresses expression of HIF-1α. Expression of HIF-1α protein was assessed following transfection. *P<0.05 and **P<0.01. miR, microRNA; HIF-1α, hypoxia inducible factor-1α; NC, negative control.

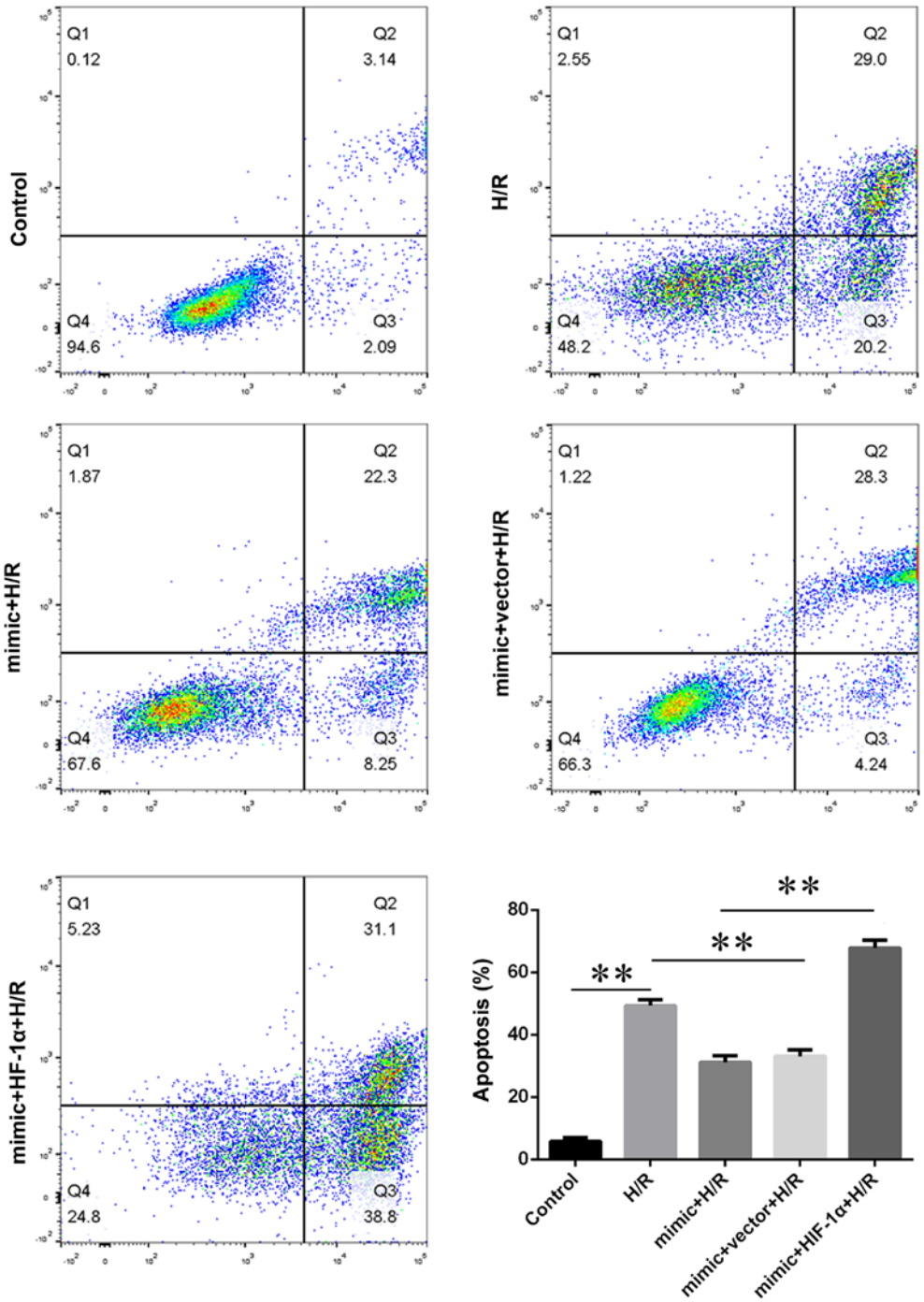

Figure 8. miR-590-3p inhibits apoptosis via HIF-1α in H/R-treated cardiomyocytes. Cell apoptosis was measured by flow cytometry. **P<0.01. miR, microRNA; HIF-1α, hypoxia inducible factor-1α; H/R, hypoxia/reoxygenation.

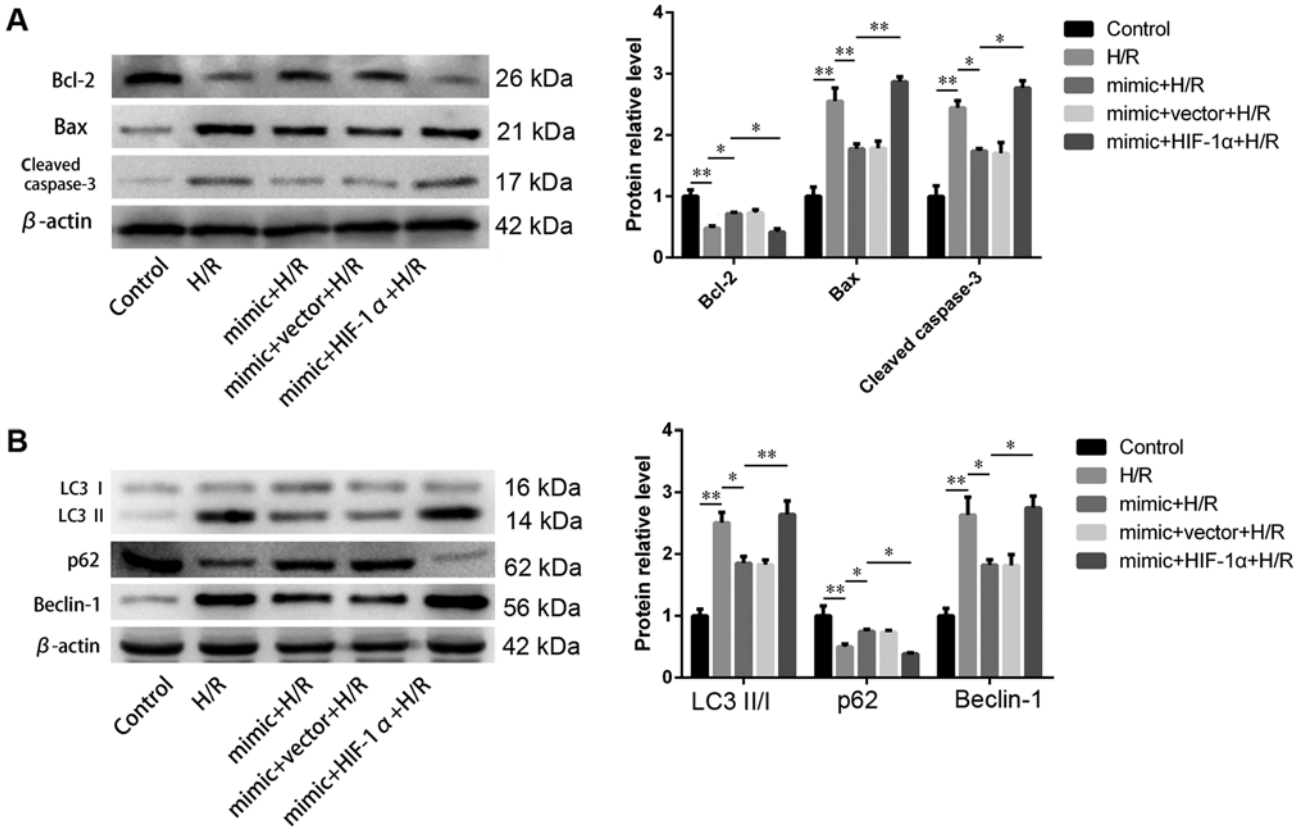

Figure 9. miR-590-3p inhibits expression of autophagy- and apoptosis-associated protein via HIF-1α in H/R-treated cardiomyocytes. Expression levels of (A) apoptosis- and (B) autophagy-associated proteins. *P<0.05 and **P<0.01. miR, microRNA; HIF-1α, hypoxia inducible factor-1α; H/R, hypoxia/reoxygenation; LC, light chain.

expression of miR-590-3p in cardiomyocytes decreased in the H/R group (Fig. 2A). miR-590-3p mimic transfection significantly improved miR-590-3p expression levels (Fig. 2B).

miR-590-3p relieves autophagy and apoptosis in primary cardiomyocytes during H/R injury. In order to evaluate the role of miR-590-3p in H/R-treated cardiomyocytes, annexin V-FITC staining and flow cytometry were used to detect apoptosis rate. The results indicated that H/R treatment induced apoptosis and this was significantly reversed by miR-590-3p mimic transfection (Fig. 3). Apoptosis- and autophagy-associated proteins were detected by western blot analysis. Levels of apoptotic protein Bcl-2 was decreased but those of Bax and cleaved caspase-3 were increased following H/R treatment. miR-590-3p transfection significantly rescued these changes (Fig. 4A), which was consistent with flow cytometry analysis. In addition, autophagy was activated following H/R treatment; this was accompanied by increased LC3II/I ratio and Beclin-1 protein levels and decreased p62 protein level. Transfection of miR-590-3p mimic significantly reversed the changes caused by H/R (Fig. 4B).

Expression of HIF-1α increases in primary cardiomyocytes following H/R treatment. In order to assess the protein expression levels of HIF-1α, western blot analysis was performed in the H/R and control groups. The results indicated that compared with the control group, the expression of HIF-1α protein was increased in the H/R group (Fig. 5).

HIF-1α is a target gene of miR-590-3p. In order to determine the target gene of miR-590-3p, bioinformatics analysis was performed using the TargetScan database. The 3'UTR region of HIF-1α gene contained a binding site for miR-590-3p. In order to verify whether miR-590-3p regulates the transcriptional activity of HIF-1α by targeting the 3'UTR of HIF-1α, luciferase reporter assay was performed. The results confirmed that HIF-1α was a direct target gene of miR-590-3p. (Fig. 6).

miR-590-3p inhibits autophagy and apoptosis via HIF-1α in H/R-treated cardiomyocytes. In order to investigate the role of HIF-1α in miR-590-3p-mediated inhibition of autophagy and apoptosis in cardiomyocytes, HIF-1α overexpression vector was constructed and co-transfected with miR-590-3p mimic. Transfection efficiency was detected by western blot analysis (Fig. 7).

Flow cytometric analysis revealed that HIF-1α overexpression significantly increased the apoptotic rate in the HIF-1α + mimic + H/R group compared with mimic + H/R group (Fig. 8). Compared with mimic + H/R group, the expression levels of apoptotic and autophagic proteins in the mimic + HIF-1α + H/R group were significantly increased (Fig. 9).

Discussion

The present data suggested that miR-590-3p exhibited a protective effect on cardiomyocytes following H/R treatment.

The protective effect was due to inhibition of autophagy and apoptosis. The mechanism may be mediated by inhibition of HIF-1α. The present study indicated that miR-590-3p relieved I/R injury and confirmed miR-590-3p as a potential therapeutic target for myocardial ischemia.

miRNAs are an important medium in the pathological process of various types of cardiovascular disease and may be a therapeutic target (30). One of the challenges in developing miRNAs to treat cardiovascular disease is to identify such miRNAs. Correcting dysregulated miRNAs can provide options for clinical treatment of cardiovascular disease (9). In the present study, H/R-treated cardiomyocytes were used to simulate myocardial I/R injury and it was found that expression levels of miR-590-3p were significantly decreased. Overexpressing miR-590-3p inhibited H/R-induced cardiomyocyte apoptosis. Apoptosis is a key part of myocardial I/R injury and is regulated by miRNAs (31). For example, miR-327 attenuates cardiomyocyte apoptosis induced by myocardial I/R injury (32). Cardiomyocytes are terminally differentiated cells that can cause irreversible damage to myocardium following apoptosis (33). miRNAs are involved in the induction of apoptosis during I/R injury (34). Annexin V-FITC/PI staining demonstrated that miR-590-3p mimic inhibited cardiomyocyte apoptosis following H/R, suggesting that miR-590-3p promoted cell survival. To the best of our knowledge, there are no previous reports on the protective effect of miR-590-3p in myocardial I/R injury.

Autophagy is a highly conserved catabolic process that maintains homeostasis of cells by removing damaged proteins and organelles (35). However, under pathological conditions, chronic upregulation of autophagy may lead to an imbalance of homeostatic conditions (35). In the present study, autophagy was evaluated by detecting LC3II/I ratio and Beclin-1 and p62 protein levels. Increased LC3II/I ratio is a sign of increased autophagosome formation (36). Beclin-1 is a key molecule in autophagy and is regulated by miRNAs (37). In addition, p62 has been identified as a substrate that can be degraded by interacting with LC3 via the autophagy-lysosomal pathway (38). In the present study, LC3II/I and levels of Beclin-1 increased, whereas p62 expression levels decreased in H/R-treated cardiomyocytes. Following miR-590-3p mimic transfection, LC3II and the ratio of LC3II/I decreased, Beclin-1 expression levels decreased and p62 levels increased, which indicated that autophagy increased following H/R treatment; miR-590-3p reversed the increased autophagy induced by H/R treatment.

The mechanism of miR-590-3p in alleviating myocardial I/R injury was further investigated. The target of miR-590-3p was predicted and it was confirmed that HIF-1α directly interacts with miR-590-3p. The expression of HIF-1α was increased in H/R-treated cardiomyocytes, but significantly decreased following miR-590-3p mimic transfection. These data suggest it is a promising target for miR-590-3p-mediated protection. HIF-1α is a key transcription and regulatory factor in hypoxia and is induced during hypoxia or ischemia (39). High expression levels of HIF-1α can initiate a series of events, including apoptosis (40). HIF-1α is associated with activation of autophagy. Overexpression of HIF-1α can induce autophagy in numerous types of cell (41,42). Inhibition of HIF-1α activity decreases I/R and H/R injury in rat heart and cardiomyocytes (43,44). In the present study, miR-590-3p inhibits apoptosis and autophagy by down-regulation of HIF-1α, protecting cardiomyocytes from H/R injury.

In summary, the present study suggested that increased miR-590-3p expression decreases myocardial autophagy and apoptosis by specifically targeting HIF-1α, thus decreasing myocardial I/R injury; this may provide novel therapeutic options for clinical treatment of myocardial I/R injury.

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Availability of data and materials

All data generated or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

XC and QG designed the study, NG and XY collected and analyzed the data, and confirm the authenticity of all the raw data. XL, YJ, CG and SM analyzed the data and drafted the manuscript and critically revised the manuscript for important intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal procedures complied with the United States National Institutes of Health Guide and were approved by the Animal Ethics Association of Bengbu Medical College, China (approval no. 075, 2017).

Patient consent for publication

Not applicable.

Competing interests

The authors declare they have no competing interests.

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