Overexpression of miR-200a protects cardiomyocytes against hypoxia-induced apoptosis by modulating the kelch-like ECH-associated protein 1-nuclear factor erythroid 2-related factor 2 signaling axis

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Abstract. The kelch-like ECH-associated protein 1 (Keap1)-nuclear factor erythroid 2-related factor 2 (Nrf2) signaling axis plays an important role in regulating oxidative stress in ischemic cardiomyocytes. Targeting Keap1 in order to promote Nrf2 activation is considered a potential method for protecting cardiomyocytes against ischemic injury. In recent years, microRNAs (miRNAs or miRs) have emerged as powerful tools for controlling gene expression. The present study aimed to determine whether Keap1-Nrf2 was regulated by specific miRNAs in cardiomyocytes under hypoxic conditions. We demonstrated that miR-200a was significantly downregulated in ischemic myocardial tissues and hypoxic cardiomyocytes. The overexpression of miR-200a was found to protect cardiomyocytes from hypoxia-induced cell damage and the excessive production of reactive oxygen species. Through bioinformatics analysis and a dual-luciferase report assay, miR-200a was found to interact with the 3'-untranslated region of Keap1, the native regulator of Nrf2. Reverse transcription-quantitative polymerase chain reaction and western blot analysis revealed that miR-200a negatively regulated the expression of Keap1. The overexpression of miR-200a significantly increased the nuclear translocation of Nrf2 as well as downstream antioxidant enzyme gene expression. The inhibition of miR-200a displayed the opposite effects. Restoring the expression of Keap1 significantly abrogated the protective effect of miR-200a. Taken together, these findings indicate that the suppression of Keap1 by miR-200a exerted a cardioprotective effect against hypoxia-induced oxidative stress and cell apoptosis, and suggest that the activation of Nrf2 signaling by miR-200a represents a novel and promising therapeutic strategy for the treatment of ischemic heart disease.

Introduction

Ischemia and reperfusion injury following cardiac operations or myocardial infarction is the principal reason for cardiac failure, morbidity, and mortality (1,2). However, an effective treatment for preventing myocardial ischemia and reperfusion injury remains unavailable due to the complicated underlying mechanism. Under ischemic or hypoxic conditions, the mitochondria in cardiomyocytes become vulnerable and damaged, leading to the production of excessive reactive oxygen species (ROS) (3). An increase in ROS induces lipid peroxidation, DNA oxidative damage, and cardiac cell oxidative damage, eventually leading to cell apoptosis (4). Therefore, the identification of a target capable of scavenging ROS in cardiomyocytes may provide a novel effective therapy for the management of myocardial ischemia and reperfusion injury.

Phase II detoxification enzymes and antioxidant enzymes are the critical components of cellular defensive machinery against ROS (5). These enzymes are regulated by the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) (6). The activation of Nrf2-mediated transcription is monitored by kelch-like ECH-associated protein 1 (Keap1). Under basal conditions, Keap1 interacts with Nrf2, thus facilitating ubiquitination and degradation of Nrf2. When Nrf2 is released from Keap1, Nrf2 protein translocates into the nucleus and binds to the antioxidant responsive elements to activate the transcription of antioxidant enzymes including heme oxygenase 1 (HO-1), glutathione S-transferase (GST), glutamate-cysteine ligase catalytic subunit (GCLc) and NADPH-quinone oxidoreductase 1 (NQO1) (7). The Keap1-Nrf2 signaling axis is suggested to play an important role in oxidative damage-related chronic diseases, including cardiovascular disease (8). Therefore, targeting Keap1-Nrf2 is considered an important approach for preventing myocardial ischemia and reperfusion injury.

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Abbreviations: Keap1, kelch-like ECH-associated protein 1; Nrf2, nuclear factor erythroid 2-related factor 2; miRNAs, microRNAs; ROS, reactive oxygen species; UTR, untranslated region; HO-1, heme oxygenase 1; GST, glutathione S-transferase; GCLc, glutamate-cysteine ligase catalytic subunit; NQO1, NADPH-quinone oxidoreductase 1

MicroRNAs (miRNAs or miRs) are endogenous, single-stranded, small, non-coding RNAs that are capable of modulating gene expression post-transcriptionally through binding to the 3'-untranslated region (3'-UTR) of target mRNA, leading to mRNA degradation and/or translational inhibition (9,10). Therefore, miRNAs are involved in regulating a number of cellular processes, including cell proliferation, survival and apoptosis (11). Mounting evidence indicates that the aberrant expression of miRNAs occurs in various human diseases, including myocardial infarction, and miRNAs are of therapeutic value in the management of myocardial infarction (12). The Keap1-Nrf2 signaling axis may be modulated by miRNAs (13). However, research into whether the Keap1-Nrf2 signaling axis is regulated by specific miRNAs in cardiomyocytes following myocardial ischemia and reperfusion injury is limited.

miR-200a has been reported to be involved in regulating the oxidative stress response (14,15). However, the role of miR-200a in ischemia and reperfusion-induced oxidative stress in the myocardium remains poorly understood. In the present study, we aimed to examine the role of miR-200a in cardiomyocytes under hypoxic conditions. We showed that miR-200a expression was significantly downregulated in ischemic myocardial tissues and hypoxic cardiomyocytes, indicating a potential role for miR-200a in regulating the survival of cardiomyocytes. Notably, the overexpression of miR-200a increased cell survival and inhibited cell apoptosis and ROS production in cardiomyocytes under hypoxic conditions. Further data showed that miR-200a functioned through targeting and regulating the Keap1-Nrf2 signaling axis. Taken together, these findings suggest that targeting the Keap1-Nrf2 signaling axis by miR-200a is a promising therapeutic strategy for preventing myocardial injury due to ischemia or hypoxia-induced oxidative stress.

Materials and methods

Cell culture and clinical tissue preparation. Human adult cardiomyocytes were purchased from ScienCell Research Laboratories (Carlsbad, CA, USA) and cultured in cardiomyocyte medium (ScienCell Research Laboratories) containing fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and 1% penicillin-streptomycin mix (Sigma, St. Louis, MO, USA) as per the recommended protocols. 293T cells obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS (both from Gibco) and 1/100 streptomycin-penicillin mix (Sigma). All cells were maintained in a humidified incubator containing 5% CO_2 at 37˚C. The left ventricular myocardial tissues were obtained from patients with end-stage heart failure due to ischemic cardiomyopathy at the time of heart transplantation (n=5). Left ventricular tissues obtained from the non-failing hearts of brain-dead organ donors (n=5) were used as controls. The myocardial samples were immediately frozen in liquid nitrogen and stored at -80˚C for use. The study protocol was reviewed and approved by the Institutional Human Experiment and Ethics Committee of Xianyang Central Hospital (Xianyang, China) and informed consent was obtained from all subjects.

Cell transfection and hypoxic treatment. For overexpression of miR-200a, miR-200a mimics (GenePharma, Shanghai, China) were used to transfect cells, and negative control mimics (NC mimics) were used as the control. For inhibition of miR-200a, a specific inhibitor against miR-200a was transfected into the cells, and negative control inhibitor (NC inhibitor) was used as the control. The miR-200a mimics (50 nM) and miR-200a inhibitor (100 nM) were transfected into cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The transfection efficiency was evaluated by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). In order to induce hypoxia, the cells were placed in a hypoxia chamber containing 94% N_2, 1% O_2, and 5% CO_2 at 37˚C. Cells cultured under normoxic conditions were taken as control.

RT-qPCR. Total RNA was extracted using an miRNeasy mini kit (Qiagen, Shanghai, China) according to the manufacturer's instructions. The cDNA was synthesized using a PrimeScript RT reagent kit (Takara, Dalian, China). RT-qPCR amplification was performed using a SYBR Premix Ex Taq GC kit (Takara). The following PCR cycling conditions were used: template denaturation step at 94˚C for 4 min; followed by 30 cycles of 20 sec at 94˚C, 30 sec at 55˚C, and 20 sec at 72˚C; and 72˚C for 5 min. The following primers were used: Keap1 forward, 5'-TACGATGTGGAAACAGAGACGTGGA-3' and reverse, 5'-TCAACAGGTACAGTTCTGGTCAATCT-3'; HO-1 forward, 5'-CTGGAGGAGGAGATTGAGCG-3' and reverse, 5'-ATGGCTGGTGTGTAGGGGAT-3'; NQO1 forward, 5'-TGATCGTACTGGCTCACTCA-3' and reverse, GTCAGTTGAGGTTCTAAGAC-3'; GCLc forward, 5'-TCCAGGTGACATTCCAAGCC-3' and reverse, 5'-GAAATCACTCCCCAGCGACA-3'; GST forward, 5'-CGGTACTTGCCTGCCTTTG-3' and reverse, 5'-ATTTGTTTTGCATCCACGGG-3'; β-actin forward, 5'-TGACGTGGACATCCGCAAAG-3' and reverse, 5'-CTGGAAGGTGGACAGCGAGG-3'; miR-200a forward, 5'-CGTAACACTGTCTGGTAACGATGT-3' and reverse, 5'-TGGTGTCGTGGAGTCG-3'; and U6 forward, 5'-GCTTCGGCAGCACATATACTAAAAT-3' and reverse, 5'-CGCTTCACGAATTTGCGTGTCAT-3'. Fold-changes relative to control were calculated by the $2^{-\Delta\Delta Ct}$ method.

Protein preparation and western blot analysis. The nuclear and cytosolic proteins from cells were extracted using a Nuclear Extract kit (Beyotime, Haimen, China) according to the manufacturer's instructions. Briefly, the cells were lysed in cytosolic protein extraction reagent followed by centrifugation at 12,000 rpm (4˚C) for 5 min. The supernatants containing cytosolic proteins were collected. The remaining precipitation was lysed by nuclear protein extraction reagent followed by centrifugation at 12,000 rpm (4˚C) for 10 min. The supernatants containing nuclear proteins were collected. The protein concentration was measured using a BCA kit (Beyotime). Protein extractions were separated by 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were then transferred to a polyvinylidene difluoride membrane (Millipore, Boston, MA, USA). The membrane was then blocked and probed with primary antibodies at 4˚C overnight. Subsequently, the membrane was incubated with horseradish peroxidase conjugated secondary antibodies (1:2,000; Beyotime) and detected using chemiluminescence. The following primary antibodies, which were purchased
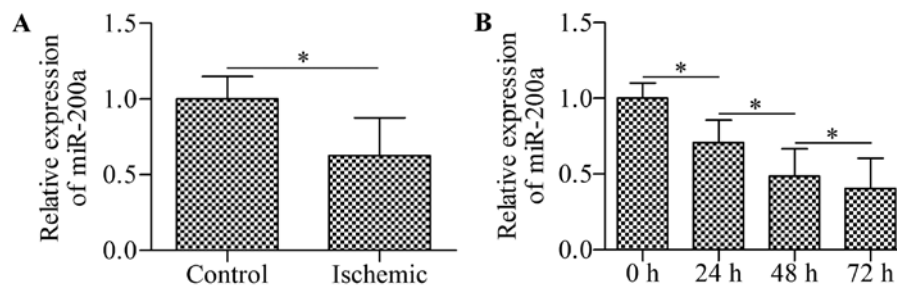
Figure 1. Expression of miR-200a in ischemic and hypoxic cardiomyocytes. (A) RT-qPCR of miR-200a expression in ischemic myocardial samples and non-failing myocardial samples. Non-failing myocardial samples were used as the control. *p<0.05. (B) RT-qPCR of miR-200a expression in cardiomyocytes under hypoxic conditions. Cardiomyocytes were subjected to hypoxia for 24, 48 and 72 h. *p<0.05.

from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), were used in this experiment: anti-Keap1 (sc-33569), anti-Nrf2 (sc-13032), anti-β-actin (sc-130656) and anti-Lamin B (sc-6216). The signal intensity of the western blots was analyzed using Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockille, MD, USA).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cell viability was detected by the MTT assay. Briefly, the cells were seeded into 96-well plates at a density of $1x10^4$ cells/well and cultured overnight. The cells were transfected with miR-200a mimics or miR-200a inhibitor and then subjected to hypoxic conditions for 48 h. Thereafter, 20 μl of MTT stock solution (Sigma) was added to each well and incubated for 4 h prior to adding 200 μl of dimethyl sulfoxide/well (Sigma). After the formazan crystals dissolved, absorbance was measured at 490 nm using a microplate spectrophotometer (Bio-Tek Instruments, Winooski, VT, USA).

Lactate dehydrogenase (LDH) assay. Cell survival was measured using an LDH assay. Briefly, the cells treated with miR-200a mimics or miR-200a inhibitor were subjected to hypoxia for 48 h. The cells were harvested and lysed using 0.2% Triton X-100 followed by centrifugation at 10,000 x g for 10 min at 4˚C. The supernatants were collected and analyzed using an LDH assay kit (Beyotime). The OD value at 490 nm was determined using a microplate reader (Bio-Tek Instruments). The lysis ratio was calculated according to the following formula: (experimental release-spontaneous release)/(maximum release-spontaneous release) x100%.

Cell apoptosis assay. Cell apoptosis was evaluated using a caspase-3 activity assay. Briefly, the cells were lysed in ice-cold lysis buffer. Following centrifugation at 10,000 x g for 1 min at 4˚C, the supernatant was collected, and the protein concentration was measured. Approximately 100 μg of protein was reacted with 5 μl of 4 mM DEVD-p-nitroanilide (pNA) substrate at 37˚C for 2 h. Finally, the levels of pNA release were quantified using a spectrophotometer (Bio-Tek Instruments).

Measurement of ROS. ROS production was detected using the 2',7'-dichlorofluorescein diacetate (DCFH-DA) method. Briefly, the cells were incubated with 50 μM DCFH-DA (Sigma) for 30 min at 37˚C in a dark place. After washing with phosphate buffer solution, the fluorescence intensity was measured in a fluorescence spectrophotometer (Bio-Tek Instruments) with an excitation wavelength of 485 nm and emission wavelength of 530 nm.

Luciferase reporter assay. The potential target genes of miR-200a were predicted by bioinformatics analysis using Targetscan (http://targetscan.org/) and MicroRNA.org - Targets and Expression (http://www.microrna.org/) databases. The 3'-UTR of Keap1 was cloned into pmirGLO dual-luciferase vectors (Promega, Madison, WI, USA) downstream of the luciferase gene. The pmirGLO-Keap1 3'-UTR constructs or relevant mutants were co-transfected with miR-200a mimics into 293T cells using Lipofectamine 2000 (Invitrogen). Following a 48-h incubation period, luciferase activity was measured using a Dual-Glo luciferase assay system (Promega).

Rescue assay. For overexpression of Keap1, the open reading frame of Keap1 cDNA without 3'-UTR was cloned into pcDNA3.0 vectors (BioVector, Beijing, China). The pcDNA3/Keap1 constructs were transfected into cells using Lipofectamine 2000 (Invitrogen) at a final concentration of 0.5 μg/μl. Empty vectors were used as a control.

Statistical analysis. All experimental data are presented as the means ± standard deviation. Statistical difference was determined by one-way analysis of variance (ANOVA) or the Student's t-test (SPSS software version 11.5; SPSS Inc., Chicago, IL, USA). When ANOVA showed statistical significance, a post-hoc Bonferroni test was conducted. A p-value <0.05 was considered to indicate a statistically significant difference.

Results

miR-200a is downregulated in ischemic myocardial tissues and hypoxic cardiomyocytes. To examine the potential role of miR-200a in ischemic heart disease, we first detected the expression of miR-200a in clinical myocardial tissues using RT-qPCR. The results revealed that miR-200a expression was significantly decreased in the ischemic myocardial tissues compared with that in the non-failing myocardial tissues (Fig. 1A). Furthermore, an *in vitro* experiment showed that miR-200a was downregulated in a time-dependent manner in cardiomyocytes exposed to hypoxia (Fig. 1B). These results indicate the important role of miR-200a in ischemic heart disease.

Overexpression of miR-200a rescues cell viability impaired by hypoxia. To deterine the biological effect of miR-200a on cell
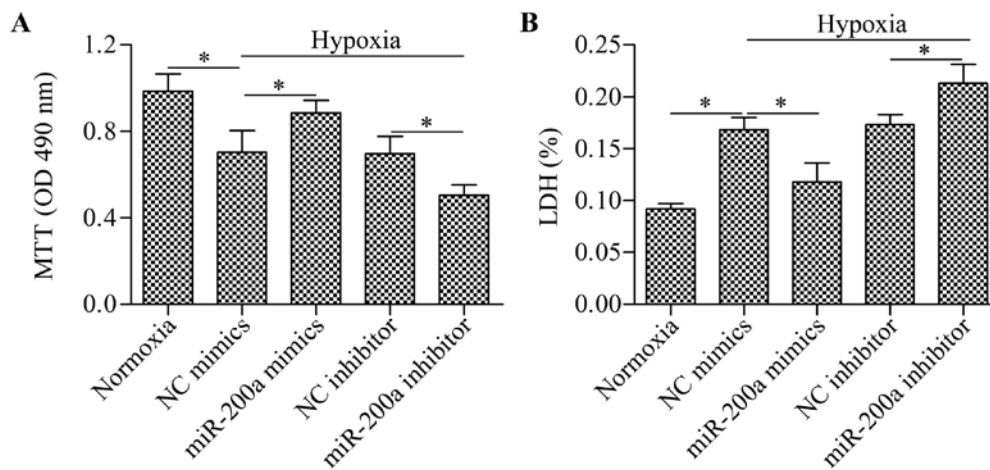
Figure 2. Impaired viability of cardiomyctes induced by hypoxia is rescued by the overexpression of miR-200a. Hypoxia-induced cell injury was detected by (A) MTT and (B) lactate dehydrogenase (LDH) assays after 48 h of exposure to hypoxia. *p<0.05.
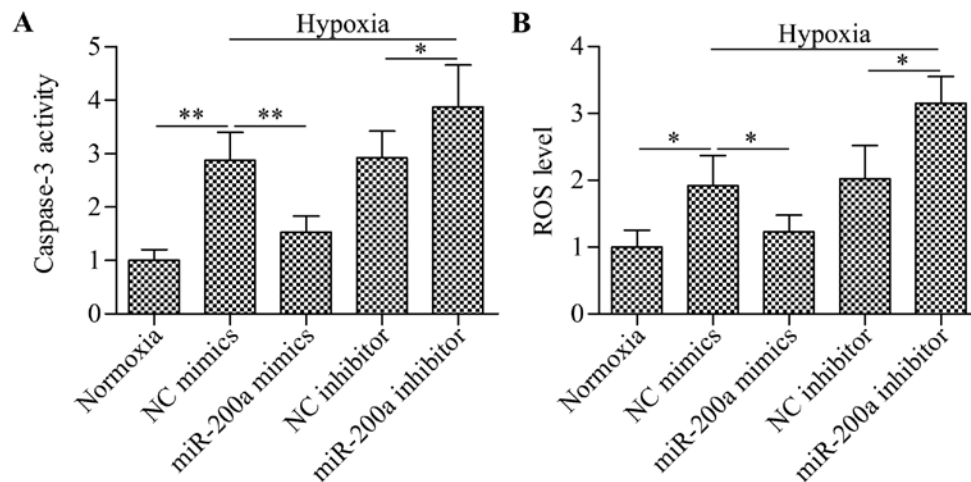

Figure 3. Overexpression of miR-200a decreases cell apoptosis and reactive oxygen species (ROS) production induced by hypoxia. (A) Cell apoptosis was detected by the caspase-3 activity assay. (B) ROS levels were measured by the DCFH-DA assay. The cells with treatment were exposed to hypoxic conditions for 48 h prior to harvesting for analysis. The data were normalized to the normoxia group. *p<0.05 and **p<0.01.

viability, we performed gain-of-function or loss-of-function experiments using miR-200a mimics or miR-200a inhibitor to overexpress miR-200a or silence miR-200a. We then detected their effects on cell viability using MTT and LDH assays. The results showed that cell viability was significantly impaired by hypoxia, but this impairment was partially rescued by miR-200a overexpression (Fig. 2). By contrast, the silencing of miR-200a by miR-200a inhibitor significantly augmented the impaired cell viability induced by hypoxia. These results suggest that overexpression of miR-200a is beneficial for cardiomyocyte survival.

Overexpression of miR-200a decreases cell apoptosis and ROS production induced by hypoxia. To further verify the biological effect of miR-200a in regulating cardiomyocyte survival, we evaluated cell apoptosis using a caspase-3 assay. The results showed that the activity of caspase-3 was significantly upregulated by hypoxia, which was significantly reduced by miR-200a overexpression or further increased by miR-200a silencing (Fig. 3A). Moreover, the excessive ROS levels induced by hypoxia was significantly decreased by

miR-200a overexpression or further increased by miR-200a silencing (Fig. 3A). These results suggest that overexpression of miR-200a improved cardiomyocyte survival by reducing cell apoptosis and ROS production under hypoxic conditions.

Keap1 is a target of miR-200a. To elucidate the underlying molecular mechanism, we performed a bioinformatic analysis to predict the possible target gene of miR-200a. Notably, we found that Keap1 contained a theoretical miR-200a binding site in the 3'-UTR (Fig. 4A). To verify this prediction, we cloned the Keap1 3'-UTR into pmirGLO reporter vectors and co-transfected miR-200a mimics with this vector in 293T cells. The results showed that miR-200a overexpression significantly decreased luciferase activity (Fig. 4B). We also detected the effect of miR-200a on the mutated constructs, in which miR-200a binding sites were mutated. The results showed that miR-200a failed to decrease the activity of the luciferase gene with mutant 3'-UTR (Fig. 4B). We further detected the direct effect of miR-200a on the mRNA and protein expression of Keap1. Both RT-qPCR and western blot analysis revealed that the transfection of cardiomyocytes
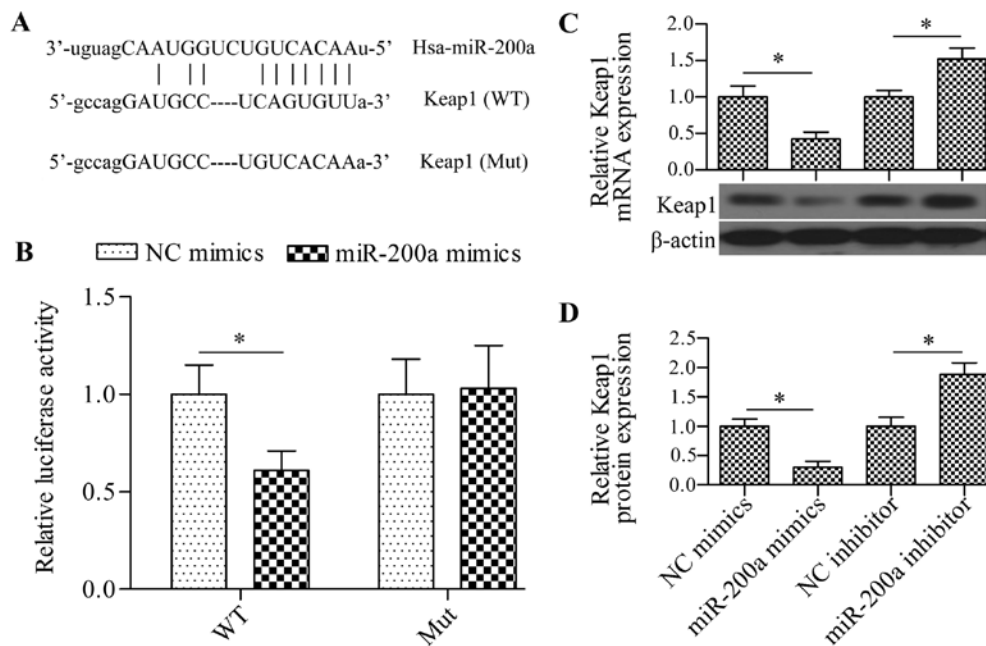
Figure 4. Kelch-like ECH-associated protein 1 (Keap1) is a target of miR-200a. (A) Sequence alignment of miR-200a and 3'-UTR of Keap1. (B) Luciferase reporter assay of miR-200a and Keap1 3'-UTR. 293T cells were co-transfected with miR-200a mimics and pmirGLO-Keap1 3'-UTR (WT) or pmirGLO-Keap1 3'-UTR (Mut). Luciferase activity was detected after 48 h of incubation. Firefly luciferase activity was normalized to *Renilla* luciferase activity. *p<0.05. (C) RT-qPCR and (D) western blot analysis were performed to detect the effect of miR-200a on the endogenous expression of Keap1. Cardiomyocytes were transfected with miR-200a mimics or miR-200a inhibitor for 48 h. Densitometric analysis of protein bands was measured using Image-Pro Plus 6.0 software. *p<0.05.
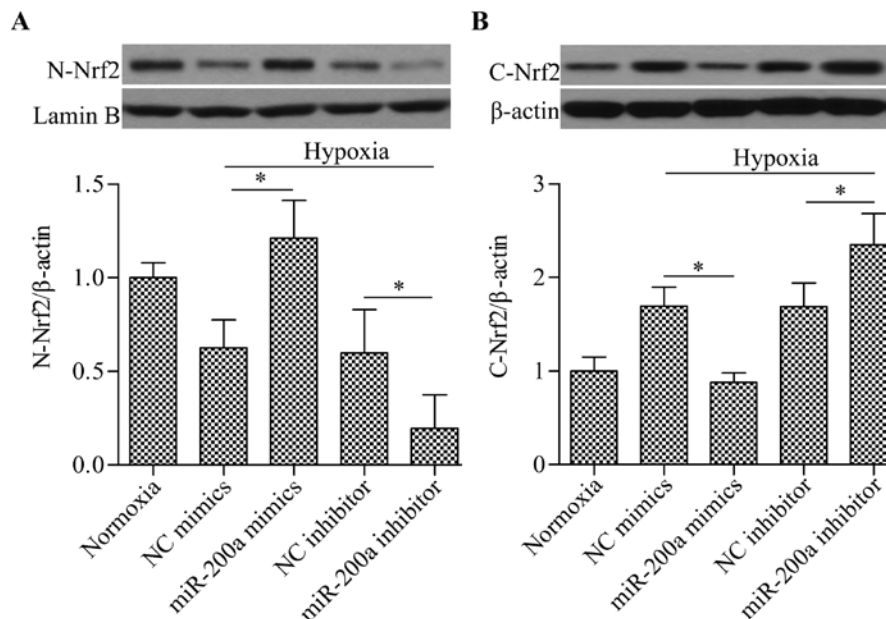

Figure 5. miR-200a affects the nuclear translocation of nuclear factor erythroid 2-related factor 2 (Nrf2). Western blot analysis of (A) nuclear and (B) cytosolic Nrf2 expression. Cardiomyocytes transfected with miR-200a mimics or miR-200a inhibitor were subjected to hypoxic conditions for 48 h. N-Nrf2, nuclear Nrf2 protein; C-Nrf2, cytosolic Nrf2 protein. Lamin B was used as the nuclear protein control. β-actin was used as the cytosolic protein control. *p<0.05.

with miR-200a mimics significantly reduced the mRNA and protein expression of Keap1, whereas the transfection of miR-200a inhibitor significantly increased the expression of Keap1 (Fig. 4C and D). These findings suggest that miR-200a inhibited Keap1 expression by directly targeting the 3'-UTR.

Overexpression of miR-200a upregulates Nrf2 nuclear translocation. Given the inhibitory effect of miR-200a on

Keap1 expression, we speculated that miR-200a may affect Nrf2 nuclear translocation. To examine this hypothesis, we evaluated nuclear and cytosolic Nrf2 protein expression by western blot analysis. The results showed that the overexpression of miR-200a significantly increased nuclear Nrf2 levels (Fig. 5A), whereas cytosolic Nrf2 levels were decreased (Fig. 5B) in the cells under hypoxic conditions. Conversely, the transfection of miR-200a inhibitor showed
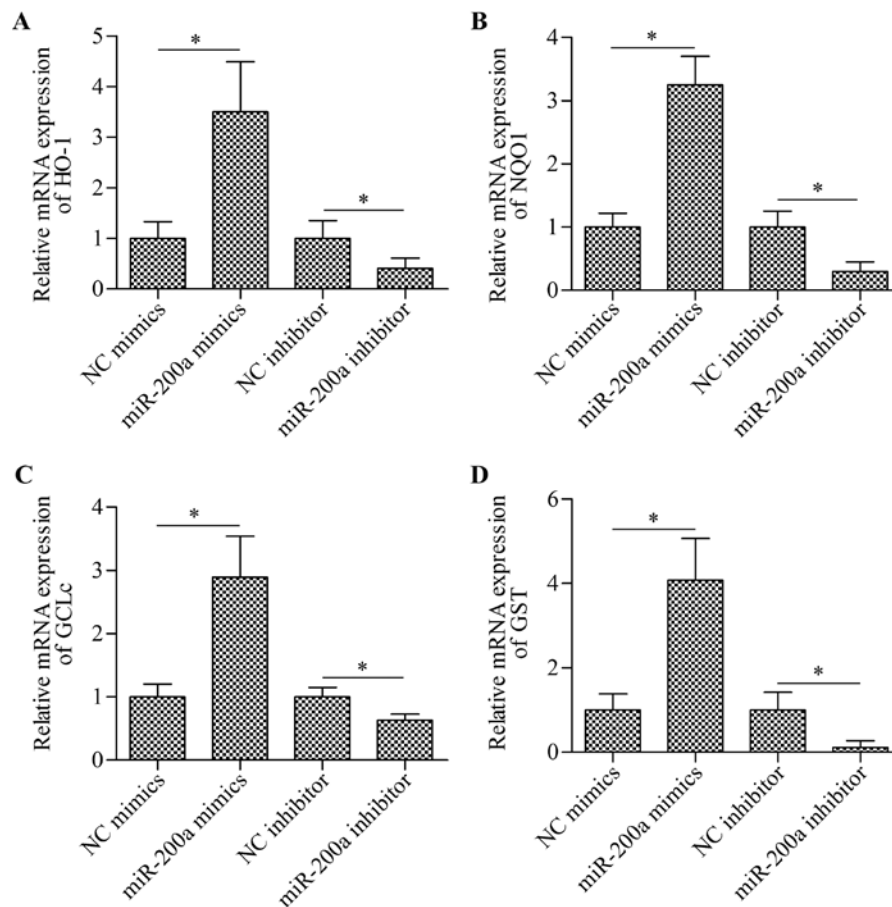
Figure 6. miR-200a regulates nuclear factor erythroid 2-related factor 2 (Nrf2) downstream signaling. The expression of (A) heme oxygenase 1 (HO-1), (B) NADPH-quinone oxidoreductase 1 (NQO1), (C) glutamate-cysteine ligase catalytic subunit (GCLc), and (D) glutathione S-transferase (GST) was detected by RT-qPCR. Cardiomyocytes transfected with miR-200a mimics or miR-200a inhibitor were subjected to hypoxic conditions for 48 h. *p<0.05.

the opposite effect (Fig. 5). Taken together, these findings suggest that the overexpression of miR-200a promotes Nrf2 release from Keap1, leading to the increased accumulation of nuclear Nrf2.

Overexpression of miR-200a increases the expression of antioxidant genes downstream of the Nrf2 signaling pathway. To further confirm the effect of miR-200a on the Nrf2 signaling pathway, we detected the effect of miR-200a on the expression of antioxidant enzymes, namely HO-1, NQO1, GCLc and GST, that are transcribed upon Nrf2 activation. The results of RT-qPCR demonstrated that the transfection of miR-200a mimics significantly increased the expression of HO-1, NQO1, GCLc and GST, whereas the transfection of miR-200a inhibitor markedly decreased the expression of these antioxidant enzymes (Fig. 6). The data indicate that the overexpression of miR-200a promotes the activation of the Nrf2 signaling pathway.

Overexpression of Keap1 reverses the effect of miR-200a overexpression on Nrf2 signaling pathway. To further verify whether miR-200a regulates the Nrf2 signaling pathway through Keap1, we performed a rescue experiment. Cells were co-transfected with miR-200a mimics and Keap1 overexpressing vectors. Western blot analysis showed that restoring Keap1 expression (Fig. 7A) significantly blocked the enhancing effect of miR-200a mimics on nuclear Nrf2

accumulation (Fig. 7B). Furthermore, the restoration of Keap1 expression blocked the inhibitory effect of miR-200a mimics on cell apoptosis (Fig. 7C) and ROS generation (Fig. 7D). Taken together, these results indicate that miR-200a regulates the Nrf2 signaling pathway, cell apoptosis and ROS levels through Keap1.

Discussion

In recent years, finding ways of overcoming the hypoxia-induced apoptosis of cardiomyocytes has remained the principal challenge in the treatment of ischemic heart disease. In this study, we demonstrated that miR-200a plays an important role in regulating cardiomyocyte survival under hypoxic conditions. We delineated that miR-200a directly targeted and inhibited Keap1, the native regulator of Nrf2. Therefore, inhibiting the expression of Keap1 with miR-200a released Nrf2, leading to the increased nuclear translocation of Nrf2 and subsequent activation of antioxidant genes. During ischemia or hypoxia, oxidative phosphorylation in mitochondria is severely impaired, leading to excessive ROS production and oxidative stress. Moreover, prolonged oxidative stress decreases the antioxidant defense ability of cells (16,17). Therefore, enhancing the Nrf2-mediated transcription of antioxidant genes in order to induce the antioxidant defense abilities of cardiomyocytes is a feasible option. Considering the regulatory effect of miR-200a
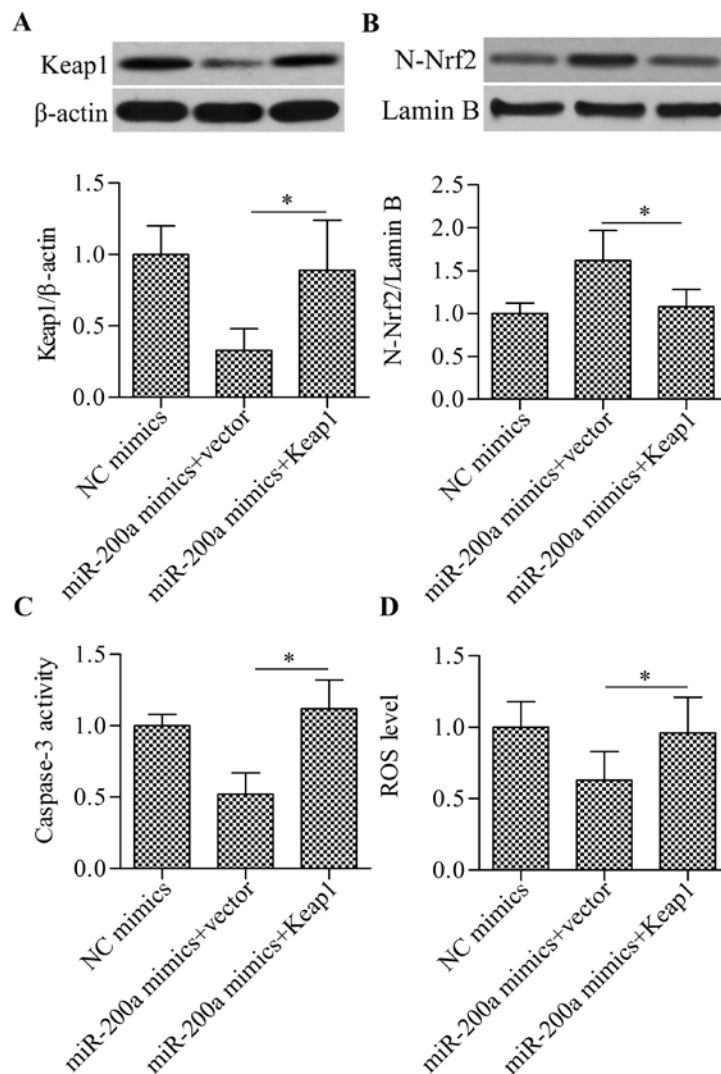
Figure 7. Restoration of Kelch-like ECH-associated protein 1 (Keap1) expression blocks the effects of miR-200a overexpression. Western blot analysis of (A) Keap1 and (B) nuclear nuclear factor erythroid 2-related factor 2 (Nrf2) protein expression. (C) Cell apoptosis was detected by the caspase-3 activity assay. (D) Reactive oxygen species (ROS) levels were measured by the DCFH-DA assay. Cells were co-transfected with miR-200a mimics and pcDNA3/Keap1 expression vector without the 3'-UTR region for 48 h under hypoxic conditions. *p<0.05. miR-200a mimics + vector, cells were co-transfected with miR-200a mimics and empty pcDNA3.0 vectors. miR-200a mimics + Keap1, cells were co-transfected with miR-200a mimics and pcDNA3/Keap1 vectors.

on the Keap1-Nrf2 signaling axis, miR-200a may serve as a critical molecular target for the treatment of ischemic heart disease.

Nrf2 is the master regulator of cellular adaptation to oxidative stress that regulates a series of antioxidant enzymes (18,19). The overexpression of Nrf2 has been demonstrated to protect against oxidative stress and cell apoptosis following ischemia and reperfusion injury (20,21). Increasing evidence indicates that Nrf2 is the target of numerous antioxidant reagents through which antioxidant reagents protect cells against oxidative stress and cell apoptosis induced by external insults (22-25). The Nrf2 signaling pathway plays an important role in cardiovascular disease (8). Acute exercise stress may promote antioxidant mechanisms in the myocardium through the activation of Nrf2 signaling (26). The apoptosis of cardiomyocytes induced by exposure to high glucose levels is inhibited by diallyl trisulfide through Nrf2 signaling (27). The activation of Nrf2 signaling may effectively attenuate the apoptosis of cardiomyocytes under ischemic or hypoxic conditions (28-31). The Keap1

protein is the native regulator of Nrf2 that blocks Nrf2 nuclear import and Nrf2 signaling activation. Thus, the inhibition of Keap1 increases Nrf2 activity (32,33). In this study, we have shown that the inhibition of Keap1 by miR-200a significantly increased the nuclear translocation of Nrf2 and subsequent downstream gene transcription, thus leading to decreased ROS production and cell apoptosis induced by hypoxia in cardiomyocytes. These findings further support the important role of Nrf2 signaling in protecting cardiomyocytes against hypoxia.

The miRNAs represent novel tools which are capable of interrupting the Keap1-Nrf2 interaction. miR-141 is reported to target Keap1 and regulate Nrf2 signaling in renal oxidative stress induced by hyperglycemia (34). Similarly, Shi *et al* reported that miR-141 targeted Keap1 to regulate Nrf2 signaling thereby conferring drug resistance to 5-fluorouracil on hepatocellular carcinoma cells (35). The downregulation of miR-29 promotes Keap1 expression that reduces Nrf2, leading to high glucose-induced apoptosis (36). miR-200a is also reported to target Keap1 and regulate the Keap1-Nrf2 signaling pathway

in breast cancer cells (37) and in hepatic stellate cells (38). However, whether specific miRNAs target Keap1-Nrf2 in cardiomyocytes has not been well studied. Herein, for the first time to the best of our knowledge, we demonstrated that Keap1 was targeted and regulated by miR-200a in cardiomyocytes using a luciferase reporter assay, RT-qPCR, and western blot analysis. The overexpression of miR-200a was shown to activate Nrf2 signaling through inhibiting Keap1, whereas the restoration of Keap1 expression significantly abrogated the effect of miR-200a overexpression on Nrf2 activation-mediated cell protection against hypoxia. Various studies also showed that Nrf2 may be targeted and regulated by specific miRNAs. Higher erythrocytic miR-144 expression results in decreased Nrf2 expression through targeting the 3'-UTR of Nrf2, leading to impaired oxidative stress tolerance in erythrocytes (39). Furthermore, miR-28 and miR-93 are reported to interact with the 3'-UTR of Nrf2 (40,41). These findings support that miRNAs are promising, novel tools for modulating the Keap1-Nrf2 signaling pathway.

In the present study, we have demonstrated that miR-200a is significantly downregulated in ischemic myocardial tissues and hypoxic cardiomyocytes. Hypoxic/ischemic stress has been suggested to contribute to the major elements for epigenetic programming such as DNA methylation (42). Hypermethylation of the promoter miR-200a has been observed in cancer cells grown under hypoxic conditions accompanied by a significant decrease in miR-200a expression (43). These findings raise the possibility that the promoter of miR-200a may be hypermethylated by hypoxic/ischemic stress which results in the decreased expression of miR-200a in ischemic myocardial tissues and hypoxic cardiomyocytes. Mounting evidence indicates that the overexpression of miR-200a promotes the pro-survival signaling pathways and dampens the pro-apoptotic genes (44,45). Notably, the high expression of miR-200a is reportedly associated with extended survival of cardiomyocytes, implying that miR-200a is beneficial for cardiomyocyte survival (46). We found that the overexpression of miR-200a protected cardiomyocytes against hypoxia-induced oxidative stress and apoptosis. The advantageous effect of miR-200a was due to its inhibitory effect on Keap1 through directly targeting the 3'-UTR of Keap1, leading to the activation of the Nrf2 signaling pathway. Taken together, these findings suggest that the overexpression of miR-200a protects cardiomyocytes against hypoxia-induced apoptosis by modulating the Keap1-Nrf2 signaling pathway, and this representing a novel strategy for the treatment of ischemic heart disease.

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