Original Article

Downregulation of miR-34a attenuates myocardial ischemia/reperfusion injury by inhibiting cardiomyocyte apoptosis

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Abstract: MicroRNAs have been shown to play critical regulatory roles in various ischemia/reperfusion (I/R) injury. However, its role in myocardial I/R injury remains largely unknown. The aim of this study was to investigate the role of microRNA-34a (miR-34a) in the development of myocardial I/R injury. Rat myocardial I/R model was established, followed by PCR array analyses to investigate the expression alterations of miRNAs in the hearts of rats. H9c2 cardiomyocytes were subjected to hypoxia/reoxygenation (H/R), and lactate dehydrogenase (LDH) release, cell viability and rat cardiomyocyte apoptosis were measured as well as miR-34a expression. Knockdown experiments were performed to determine the effects of manipulating miR-34a on apoptotic responses. Our results revealed that miR-34a was significantly up-regulated in I/R group compared with sham group. Inhibition of miR-34a promoted cell proliferation, reduced LDH release and apoptosis which were accompanied by a significant decrease in caspase-3 activity. Furthermore, Bcl-2, a well know anti-apoptotic gene was identified to be a functional target of miR-34a. Taken together, knockdown of miR-34a mediates H/R-induced apoptosis in cardiomyocytes by targeting Bcl-2 and represents a potential target for prevention of myocardial I/R injury.

Keywords: Ischemia/reperfusion (I/R) injury, miR-34a, hypoxia, apoptosis, Bcl-2

Introduction

Heart transplantation is the best option for long-term survival for end-stage heart failure (HF) patients. Up to now, more than 116,000 heart transplantations have been performed worldwide [1, 2]. Ischemia reperfusion (I/R) injury is an unavoidable event occurring during heart transplantation, leading to graft failures and lower long-term survival rate of the recipient. Thus, there is an urgent need for well-tolerated therapies with the ability to significantly attenuate adverse heart transplantation and improve function of the failing heart [3-5]. It is important to explore new alternative mechanisms involved in I/R injury during heart transplantation.

The pathophysiological mechanisms of myocardial I/R are complicated. Apoptosis plays an important role in different kinds of I/R injury, including myocardial I/R. Accumulating evidence has demonstrated that miRNAs have crucial roles in various cellular and biological processes, including cell growth, differentiation, migration and especially apoptosis [6-10]. MicroRNAs (miRNAs) are highly conserved, small, noncoding RNAs consisting of about 18-24 nucleotides in length, which regulates gene expression by targeting mRNAs and triggering either translation repression or RNA degradation [11-13]. Several studies have demonstrated that miRNAs are vital regulators of signaling pathways involved in I/R injury [14-17]. However, whether miRNAs are involved in the apoptosis induced by myocardial I/R is few studied.

In this study, we examined alterations in miRNA expression levels in the hearts in an I/R rat model using PCR array analyses, and miR-34a was significantly altered by hypoxia. Furthermore, it was demonstrated that knockdown of miR-34a could attenuate myocardial I/R injury by targeting Bcl-2 in vitro. Our data suggest that miR-34a may provide a new thera-
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Materials and methods

A rat model of myocardial I/R injury

In order to study the microRNA expression changes in myocardial I/R injury, we applied a rat myocardial I/R injury model. In brief, 6-10-week-old male Sprague-Dawley rats (weighting 270-320 g) were used to set I/R models and the Sham controls. Rats were anesthetized with ketamine (80 mg/kg intraperitoneally) and xylazine (5 mg/kg intraperitoneally), placed in a supine position. Under sterile conditions, the animals were ventilated with room air using a rodent respirator. The arterial blood pressure (BP) was recorded via femoral artery catheterization with a bio-signal recorder, and the standard limb lead I electrocardiogram were synchronously monitored until the end of the experiment. Sham-operated rats served as controls. Sham operation involved an identical procedure, except the suture was passed around the vessel without left anterior descending coronary artery occlusion. The procedures for the experiments and animal care were approved by the institutional animal care and the ethics committee of Zhongshan Hospital, Fudan University, and all animals received humane care in compliance with The Guide for the Care and Use of Laboratory Animals, published by National Institutes of Health.

miRNA PCR array profiling analysis

The Rat Apoptosis miRNA PCR Array (Qiagen, Hilden, Germany) was used to study the expression of microRNA in rat myocardial I/R injury. Briefly, cDNA was prepared using miScript II RT Kit with miScript HiSpec Buffer (Qiagen, Hilden, Germany), incubated at 37°C for 60 min, then at 95°C for 5 min. MiScript SYBR Green PCR Kit (Qiagen, Hilden, Germany) was used for the real-time PCR reactions on the miScript miRNA PCR Array. Add PCR mix to miScript miRNA PCR Array, Perform real-time PCR, Analyze results using miScript miRNA PCR Array data analysis tool. The amplification data (fold changes in the threshold cyte values of all the genes) were analyzed by the $2^{\Delta\DeltaCT}$ method. According to the manufacturer’s protocol, 84 miRNAs known or predicted to alter in expression during apoptosis was profiled.

Total RNAs extraction and real-time (RT) PCR

Total RNA were extracted from cells using Trizol (Invitrogen, CA, USA) according to the manufacturer's instructions. Reverse-transcribed complementary DNA was synthesized with the PrimeScript RT reagent kit (TaKaRa, Japan). Mature miR-34a primers were synthesized by GenScript Co. Ltd (China). The primers were as follows: miR-34a (forward, 5’-TGCCTGGCG-AGTGTCTTAGGT-3’; reverse, 5’-CAAGTCCAGGTTCGGAGGATT-3’). Real-time quantitative PCR was performed on a Rotor-Gene 3,000 real-time DNA detection system (Corbett Research, Australia) using SYBR Green (Qiagen, Hilden, Germany). All samples were analyzed in duplicate, including no-template controls. The relative expression levels of miRNAs and the potential target genes of miR-34a were determined by the standard curve method, respectively.

Transfection H9c2 cells and in vitro I/R model

The H9c2 rat ventricular cell line was purchased from the cell bank of the Chinese Academy of Sciences. Cells were seeded at a concentration of $2 \times 10^4$ cells/cm$^2$, and cultured with Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich Corp, USA) containing 10% (v/v) fetal bovine serum (FBS, HyClone Laboratories, USA) in a humidified atmosphere of 5% CO$_2$ at 37°C. MiR-34a mimic, miR-34a inhibitor and the corresponding negative control (miR-NC) were purchased from Shanghai GenePharma Co. Ltd. (Shanghai, China). MiR-34a mimic or inhibitor was transfected into cells using Lipofectamine 2000 (Invitrogen, CA, USA) following the manufacturer’s instructions.

Hypoxia was induced by exposing cells to 1% O$_2$-94% N$_2$-5% CO$_2$ for 24 h using a modular incubator (Model 3131, Forma Scientific, Marietta, OH, USA). Cells cultured under a normoxic atmosphere served as a control. Cells were serum deprived for 12 h for synchronization before the onset of hypoxia, and maintained in serum-free DMEM during hypoxia.

Cell viability

H9c2 cardiomyocyte survival and structural integrity after hypoxia was evaluated by measuring cell viability. Cell viability was examined via a MTT assay according to the manufacturer's instructions (Roche Applied Science). Briefly, approximately $1 \times 10^5$ cells were seed-
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ed in a 96-well culture plate for 24 h. At various time points, MTT (Amresco, USA) was added to the medium at a final concentration of 0.5 mg/ml for the last 12 h of the experiment. The resulting formazan crystals were dissolved in dimethyl sulfoxide, the absorbance was measured on an Optima microplate reader (Molecular Devices, Sunnyvale, CA) at a wavelength of 570 nm with background subtracted at 690 nm.

Lactate dehydrogenase (LDH) release assay

Lactate dehydrogenase activity in the culture media was conducted with the LDH detection activity assay kit (Sigma, St. Louis, MO) at 450 nm according to the manufacturer’s instructions. LDH catalyzes the oxidation of L-lactate to pyruvate, and reduction of nicotinamide adenine dinucleotide (NAD) to NADH. The reduction of NAD is accompanied by the decrease of light absorbance at 340 nm. One unit of LDH activity is defined as a decrease of 1 U per min in the light absorbance at 340 nm using a path length of 1 cm. The reading of LDH was normalized to the amount of protein.

Analysis of cell apoptosis

H9c2 cells were trypsinized and washed twice with phosphate buffered saline (PBS). Cells (4 × 10^5/ml) were collected with 1 mL PBS and centrifuged at 1000 rpm for 10 min. The cells were suspended in 500 μL of Binding Buffer and mixed with 5 μL Annexin V (Bio-Science, Co. Ltd, Shanghai, China) and 5 μL PI (Bio-Science, Co. Ltd., Shanghai, China). After incubation at RT in the dark for 5 min, samples were analyzed by a FACS Aria flow cytometry (BD Biosciences, San Jose, CA, USA).

Western blot analysis

Total protein samples were isolated from H9c2 cells. Cells were washed twice with ice-cold PBS, collected in lysis buffer (Thermo Scientific, Rockford, IL, U.S.A), supplemented with a protease inhibitor mixture (Sigma, St. Louis, MO), and the lysates were centrifuged. Equal amounts of protein from each sample were separated by 12% SDS-PAGE (Bio-Rad, Hercules, USA). After electrotransferring onto nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ), the blots were incubated overnight at 4°C with the following primary antibodies against Bcl2 (Abcam), followed by incubation in the corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies (Abcam, Cambridge, UK). β-Actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as the loading control. The images were captured using a BioSpectrum-410 multispectral imaging system. Immunoreactive bands were visualized by use of an enhanced chemiluminescence kit (Millipore, USA) and quantified by use of Image-Pro Plus 6.0.

Luciferase assay

To examine whether miR-34a regulates the expression of Bcl-2, a predicted target of miR-34a, the dual luciferase psiCheck2 reporter plasmid (Promega, Madison, WI, USA) was used to generate the reporter plasmid harboring Bcl-2 3’UTR. Briefly, 3’UTR containing the putative miR-34a binding site was amplified from rat genomic DNA by PCR, using Xho1 and Not1 primers (left Xho1 primer: CTCGAGCGGAAGCTATGGACGTTCAG, right Not1 primer: GCGGCCGCCATTTGCAACTGGGATGGTTCACCAGT), the PCR product was digested with Xho1 and Not1, followed by insertion into the multiple cloning region located at the 3’UTR of the synthetic Renilla luciferase gene within the psiCheck2 plasmid. The psiCheck2 plasmid also contains a synthetic firefly luciferase gene that serves as the transfection Control. The H9c2 cells were co-transfected with the psiCheck2 vector containing Bcl-2 3’UTR and miR-34a mimic or miR-34a inhibitor using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), and the co-transfection with non-targeting negative control RNA was performed as control. The cells were harvested 24 h after transfection, and luciferase activity was measured with a dual luciferase reporter assay kit (Promega, Madison, WI, USA) on a luminometer (Lumat LB9507).

Statistical analysis

All statistical analyses were carried out using the SPSS 11.0 statistical software package. All the data are expressed as mean ± standard deviation (S.D) from three independent experiments. The differences between two experimental conditions were compared on a one-to-one basis using two-tailed Student’s test. A value of P < 0.05 was considered statistically significant.
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Results

MiRNA expression signatures in I/R injury rat hearts

Apoptosis miRNA PCR array analysis showed that a total of 20 out of 84 miRNAs were identified as being differentially expressed within ischemic myocardium rats, compared to the normal myocardium of sham-operated rats. Among these 20 specific miRNAs, miR-34a, -122, -155, -199a, -128a, -200c, -133b, -10a, -149a and -15a were up-regulated, whereas miR-125b, -222, -184, -27b, -30a, -7a, -25, -378, -9 and -10a were down-regulated in the ischemic myocardium. Among the dysregulated miRNAs, miR-34a is the most up-regulated (Figure 1A), then, miR-34a was selected for further studies. To verify PCR array analysis finding, we validated miR-34a expression in eight myocardial I/R injury rats. qPCR results showed that miR-34a expression was also upregulated in the ischemic myocardium of rats compared to the myocardium of sham-operated rats (Figure 1B). Taken together, these data suggest miR-34a may play an important role in the progression of myocardial I/R injury.

Hypoxia model of cultured rat H9c2 cardiomyocyte

H9c2 cell I/R injury model has been widely used to mimic myocardial I/R injury in vivo [18]. In our study, we established H9c2 cells I/R injury model, determine the optimal hypoxia time; we researched the effect of hypoxia time on LDH release, cell viability, cell apoptosis and caspase-3 activity. Compared with the normal cultured H9c2 cells, the level of LDH release was significantly increased after hypoxia, reached the largest increase LDH release in 24 h after hypoxia (Figure 2A). The simulated ischemia induced a time-dependent decrease of cell activity, the time-course changes showed a largest decrease in cell viability at 24 h after hypoxia.
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Figure 2. Hypoxia model of cultured rat H9c2 cardiac myocytes. Cells were serum deprived for 12 h before the onset of hypoxia, and maintained in serum-free DMEM during hypoxia. A. The time-course changes of LDH releases in H9c2 cells exposed to hypoxia. B. The time-course changes of cell viability in H9c2 cells exposed to hypoxia. C. The time-course changes of cell apoptosis in H9c2 cells exposed to hypoxia. D. The time-course changes of caspase-3 activity in H9c2 cells exposed to hypoxia. E. The time-course changes of miR-34a expression levels in H9c2 cells exposed to hypoxia. Data are presented as the means ± SD from three independent experiments. (*P < 0.05, **P < 0.01 vs. control).

Additionally, the apoptosis rate in the ischemic myocardium was 14.9% in 12 h, and 20.37% in 24 h, as determined by the Flow Cytometry. By contrast, the apoptosis rate was 3.79% in normal myocardium (Figure 2C).

Caspase-3 activity was significantly increased in H9c2 cells at 24 h of hypoxia when compared with the normal cultured H9c2 cells (Figure 2D). Therefore hypoxia of 24 h was selected for the following functional experiments in H9c2.
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In accordance with the changes of miR-34a in adult cardiomyocytes subjected to ischemia, miRNA-34a expression was down-regulated as well in cultured rat H9c2 cardiomyocytes exposed to hypoxia. The time-course changes showed a largest increase in miR-34a expression at 24 h after hypoxia (Figure 2E).

**MiR-34a inhibitor suppressed the apoptosis of H9c2 cells induced by I/R**

In cultured H9c2 cells, transfection of miR-34a inhibitor significantly decreased the level of miR-34a, transfection of negative control RNA not affect the level of miR-34a. The result showed that miR-34a inhibitor significantly suppressed miR-34a expression (Figure 3A). The level of LDH release was relatively low in normal cultured H9c2 cells, which was not affected by the negative control RNA or miR-34a inhibitor transfection. As shown in Figure 3B, exposure to hypoxia for 24 h greatly increased LDH release in blank cells, indicating damage to cell membrane integrity. However, the miR-34a inhibitor induced a decrease of LDH release in hypoxic H9c2 cells (P < 0.01). As determined by the MTT assay, negative control RNA or miR-34a inhibitor did not affect cell viability in normoxic cultures (Figure 3C). Cell viability was significantly inhibited under hypoxia in hypoxic H9c2 cells, while transfected with the miR-34a inhibitor, the viability was significantly increased (P < 0.01). The result showed the ability of miR-34a deficiency to reduce cell...
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Previous studies demonstrated that apoptosis played an important role in myocardial IR injury. Thus, we carried out Annexin V/PI assay to detect apoptosis. We found that apoptosis was markedly enhanced in cultured H9c2 cells under hypoxia compared with normoxic cells. However, after transfected with miR-34a inhibitor resulted in a much lower rate of apoptosis in hypoxia cultured H9c2 cells transfected with the miR-34a inhibitor (Figure 3D). The result showed miR-34a inhibitor effectively inhibited H9c2 cells apoptosis during hypoxia. No changes of caspase-3 mRNA were detected in normoxic H9c2 cells transfected with the negative control RNA or miR-34a inhibitor. Hypoxia increases the activity of caspase-3; it was significantly suppressed in hypoxia cultured H9c2 cells transfected with the miR-34a inhibitor (Figure 3E).

**Bcl-2 is a direct target of miR-34a in H9c2 cells**

Bcl-2 is a critical molecule for the regulation of apoptosis. Binding sites for miR-34a in the 3'UTR of Bcl-2 were identified by TargetScan and RNAhybrid algorithms (Figure 4A). To experimentally demonstrate that Bcl-2 is a direct target of miR-34a, we have identified a putative miR-34a-binding site (ACUGCC) in the
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3'UTR of Bcl-2 mRNA. Bcl-2 wild-type or mutant-type 3'UTR was subcloned into a luciferase reporter vector and transfected with miR negative control (NC), miR-34a mimic, inhibitor NC and miR-34a inhibitor into the H9c2 cells. Luciferase reporter assays showed that miR-34a mimic significantly inhibited luciferase activity of the wild-type Bcl-2 3'UTR by about 80% relative to the mimic control, whereas co-transfection with miR-34a inhibitor significantly increased luciferase activity by about 5 times relative to the inhibitor NC (P < 0.01) (Figure 4B). Transfection of the mutant Bcl-2 3'UTR with the same constructs had no effect on luciferase activity, confirming that Bcl-2 is a direct target of miR-34a. To examine further whether miR-34a modulated expression of Bcl-2 in H9c2 cells, H9c2 cells were transfected with miR NC, miR-34a mimic, inhibitor NC and miR-34a inhibitor. The results showed that transfection of the miR-34a mimic significantly suppressed protein expression of Bcl-2. In contrast, transfection of the miR-34a inhibitor significantly increased protein expression of Bcl-2 (Figure 4C). Based on these results, we concluded that miR-34a specifically suppressed Bcl-2 protein synthesis in H9c2 cells.

Discussion

This study has produced two major findings. First, miR-34a was a most obvious up-regulated miRNA in rats myocardial I/R injury model. Second, inhibition of miR-34a significantly enhanced cell viability, reduced LDH release, suppressed caspase-3 activity and inhibited apoptosis. Our results showed a therapeutic effect of miR-34a inhibition by promote upregulation of Bcl-2 in I/R injury.

I/R injury is considered to be a major mechanism for heart transplant failure [19, 20]. Thus, it is important to understand molecular mechanisms of I/R injury for the development of therapies against I/R injury, outcome of heart transplant could also guide the development of better preservation strategies to increase donor heart pools. Apoptosis plays a key role in different kinds of I/R injury, including myocardial I/R injury. More recent studies have demonstrated that cardiomyocyte apoptosis contributes to the process of cardiac damage during I/R injury, which causes the loss of cardiomyocyte volume and the subsequent cardiac dysfunction. Thus, inhibition of apoptosis may have promise as a therapeutic strategy for ischemia-reperfusion injury [21]. For example, febuxostat provides cardioprotection following I/R-induced myocardial injury by reducing ROS generation and mitochondrial apoptosis [22]. The miR-195 expression was upregulated in myocardial I/R injury and miR-195 overexpression promote cardiomyocyte apoptosis by targeting Bcl-2 and inducing mitochondrial apoptotic pathway [23]. In this study, we establish two myocardial I/R injury model by H/R stimulation. As we expected, the cardiomyocytes apoptosis was increased after H/R treatment. Interestingly, miR-34a expression was upregulated in rat model and cell model. However, whether miR-34a is involved in I/R-induced apoptosis have not been explored.

A growing body of evidence shows that miRNAs play a pivotal role in myocardial I/R injury [24-28]. For example, miR-21 had a protective effect on I/R by reducing cardiac cell apoptosis via its target gene PDCD4 [29]. Overexpression of miR-22 provides cardiac protection against I/R induced cardiomyocytes apoptosis by directly targeting CBP [30]. Upregulation of miR-25 inhibits cerebral I/R injury-induced apoptosis through downregulating Fas/FasL [31]. miR-128 inhibitor attenuated I/R injury-induced cardiomyocyte apoptosis by the targeted activation of PPARG signaling [32]. In our studies, apoptosis microRNA PCR array analysis showed that 20 miRNAs were identified as being differentially expressed, among these 20 specific miRNAs, miR-34a is the most upregulated in all of miRNA. Furthermore, miR-34a is crucially involved in apoptotic pathways, as well as disorders characterized by abnormal apoptosis. However, whether miR-34a is involved in I/R-induced cardiomyocyte apoptosis was not clear. In the present study, we found that inhibition of miR-34a by miR-34a inhibitor significantly enhanced cell viability, reduced lactate dehydrogenase release, suppressed caspase-3 activity and inhibited apoptosis in the established H9c2 hypoxia model. Our findings reveal that miR-34a may be a potential drug target for treating myocardial I/R injury.

Bcl-2, one of anti-apoptosis genes, is a crucial controller of the mitochondrial pathway [33, 34]. XF Chen et al. reported that miR-744 targeted on Bcl-2, which led to the inactivation of
apoptosis signaling and the cell proliferation of cervical cancer cells, ameliorating cervical cancer growth and progression [35]. It is reported that expression of miR-34a was markedly elevated in association with increased apoptosis of CEP chondrocytes, and that LNA modified miR-34a antisense can promote upregulation of Bcl-2 in chondrocytes to prevent apoptosis [36]. However, the significance of Bcl-2 on apoptosis induced by miR-34a in myocardial I/R injury is unknown. In this study, bioinformatics target prediction identified a potential miR-34a binding site within the 3'UTR of Bcl-2 gene. Luciferase activity analysis confirmed the prediction that miR-34a directly repress Bcl-2. Furthermore, the protein level of Bcl-2 is significantly increased by transfection of the miR-34a inhibitor, strongly indicating miR-34a inhibitor pretreatment was able to attenuate myocardial I/R injury induced Bcl-2 down regulation, as well as H9c2 cell apoptosis.

In conclusion, our findings reveal that miR-34a significantly up-regulated in rats myocardial I/R model in vivo and in vitro, and miR-34a inhibitor protected cardiomyocyte against I/R-induced apoptosis in vitro. Bcl-2 may be the target gene of miR-34a inhibitor against I/R-mediated cardiomyocyte apoptosis. Our results indicated that miR-34a/bcl-2 axes may be a potential drug target for treating cardiomyocyte I/R injury.

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Disclosure of conflict of interest

None.

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