MicroRNA-92a Inhibition Attenuates Hypoxia/Reoxygenation-Induced Myocardiocyte Apoptosis by Targeting Smad7

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Abstract

Background: MicroRNAs (miRNAs) regulate a lot of physiological and pathological processes, including myocardial ischemia/reperfusion. Recent studies reported that knockdown of miR-92a could attenuate ischemia/reperfusion-induced myocardial injury. In the present study, we examined the potential anti-apoptotic effects of miR-92a in a rat myocardiocyte cell line, and the possible role of Smad7 in such actions.

Methodology and Results: In a preliminary bioinformatic analysis, we identified SMAD family member 7 (Smad7) as a potential target for miR-92a. A luciferase reporter assay indeed demonstrated that miR-92a could inhibit Smad7 expression. Myocardial ischemia/reperfusion was simulated in rat H9c2 cells with 24-h hypoxia followed by 12-h reoxygenation. Prior to hypoxia/reoxygenation, cells were transfected by miR-92a inhibitor. In some experiments, cells were co-transfected with siRNA-Smad7. The miR-92a inhibitor dramatically reduced the release of lactate dehydrogenase and malonaldehyde, and attenuated cardiomyocyte apoptosis. The miR-92a inhibitor increased SMAD7 protein level and decreased nuclear NF-κB p65 protein. Effects of the miR-92a inhibitor were attenuated by co-transfection with siRNA-Smad7.

Conclusion: Inhibiting miR-92a can attenuate myocardiocyte apoptosis induced by hypoxia/reoxygenation by targeting Smad7.

Introduction

Myocardial ischemia/reperfusion (I/R) injury contributes to the damage after ischemic events in patients with coronary heart disease (CHD) [1,2]. I/R injury is also implicated in cardiac procedures that require cardio-pulmonary bypass, and in CHD patients receiving percutaneous coronary intervention or coronary artery bypass surgery. I/R injury is mediated by a variety of factors, including oxidative stress, intracellular Ca²⁺ overload, rapid restoration of physiological pH upon reperfusion, the mitochondrial permeability transition pore (MPTP), and exaggerated inflammation [3].

MicroRNAs (miRNAs) are a class of endogenous, small non-coding single-stranded RNAs, typically 18–24 nucleotides in length, that negatively regulate gene expression through binding to the 3' untranslated region (UTR) of target mRNAs [4]. MiRNAs play critical roles in a variety of heart diseases, including cardiac hypertrophy [5], heart failure [6], arrhythmia [7], myocardial infarction [8] and I/R injury [9]. Growing evidence also supports a pivotal role for miR-92a in multiple processes, including tumorigenesis and metastasis [10], cell proliferation and apoptosis [11]. In the study, we found that transfection with miR-92a inhibitor could attenuate myocardial injury and apoptosis induced by hypoxia/reoxygenation (H/R) in cultured rat H9c2 myocardiocytes cells. A preliminary bioinformatics analysis identified Smad7 as a target for miR-92a. Accordingly, we also examined the possible involvement of Smad7 in the protective action of miR-92a.

Materials and Methods

Cell Culture

The H9c2 cells (ventricular myocardiocyte, rat in origin; Cell Bank of the Chinese Academy of Sciences, Shanghai, China) were seeded at a density of 2×10⁴ cells/cm² in 6-well plates and cultured in Dulbecco’s modified Eagle’s medium (DMEM, Sigma, St. Louis, MO, USA) containing 10% (v/v) fetal bovine serum (FBS, HyClone, Logan, UT, USA) in a humidified atmosphere of 95% air and 5% CO₂ at 37°C.
Transient Transfection with Oligonucleotides

Transfection was carried out using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The ratio of oligonucleotide vs. the Lipofectamine 2000 transfection reagent was 1:5. MiR-92a mimic, inhibitor and matched negative control (NC) were synthesized by GenePharma, Shanghai, China. For RNA interference, cells were transiently transfected with a siRNA specific for Smad7 or NC (GenePharma). All transfections were carried out after 12-h serum starvation, and lasted for 48-h prior to the H/R experiments.

H/R in H9c2 Cardiomyocytes

Hypoxia was induced by exposing the cells to 1% O2, 94% N2, and 5% CO2 for 24 h using a modular incubator (Model 3131, Forma Scientific, Marietta, OH, USA). Reoxygenation (95% air, 5% CO2, 3% 7 Forma Scientific, Marietta, OH, USA). All experiments were performed at least three times.

Quantitative Real-time Polymerase Chain reaction (qRT-PCR)

Total RNA was extracted using Trizol reagent (Invitrogen). Bulge-loop miRNA qRT-PCR primer sets (one RT primer and a pair of qRT-PCR primers for each set) specific for miR-17, miR-18a, miR-19a, miR-20a, miR-19b and miR-92a were designed by RiboBio (Guangzhou, China). MiRNAs were reverse transcribed using the stem-loop RT primer. The primers for Smad7 were also designed by RiboBio. qRT-PCR was carried out to examine the expression of specific miRNAs or mRNA on a Rotor-Gene 3,000 using SYBR Green (Qiagen, Shanghai, China). All reactions were performed at least three times.

Western Blotting Assays

Cells were harvested in RIPA lysis buffer (Biotek Co, Beijing, China) containing 1 mM phenylmethylsulfonyl fluoride and centrifuged at 12,000×g for 15 min at 4°C. Whole cell lysate was used for SMAD7 detection. Cytosolic and nuclear fractions were prepared using standard nuclear and cytoplasmic extraction reagents (Thermo Scientific, Rockford, IL, USA). Protein concentration was measured using the Bio-Rad method. Samples (20 μg protein) were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane. The membranes were blocked with 5% non-fat milk in TBST buffer (100 mM NaCl, 10 mM Tris-HCl, pH 7.4, 0.1% Tween-20) for 1 h prior to incubation with a primary antibody against SMAD7 (ab90085; Abcam; Cambridge, MA, USA) overnight, and then stained with fluorescent-conjugated secondary antibody for another 60 min. Cells were mounted in Vectashield mounting medium containing 4′,6′-diamidino-2-phenylindole (DAPI) to visualize nuclei. Images were captured using a fluorescence laser scanning confocal microscope (FV1000, Olympus, Tokyo, Japan).

Statistics and Data Analysis

All data are expressed as the mean±SEM. Comparisons between groups were made by one-way analysis of variance or two-tailed student's t-test. Differences were considered statistically significant at P<0.05. SPSS software version 19.0 (SPSS, Chicago, IL, USA) was used for data analysis. All experiments were performed at least three times.
Results

MiR-17-92 Expression Profiles in H/R H9c2 Cardiomyocytes

In our previous study [12], we found that miR-17, miR-19a, miR-20a, miR-19b and miR-92a, but not miR-18a, were highly expressed in the heart of C57BL/6 mice. In the current study, the expression of the miR-17-92 cluster was up-regulated in H/R H9c2 cardiomyocytes: the expression of miR-92a was significantly up-regulated by 2.78-fold over the control ($P < 0.01$ vs. control) (Figure 1). Based on the most remarkable change in response to hypoxia/reoxygenation as reflected by qRT-PCR, miR-92a was selected for subsequent experiments.

Figure 2. Gene levels in normoxic H9c2 cardiomyocytes transfected with miR-92a inhibitor (A) or siRNA-Smad7 (B). H9c2 cells were transfected with miR-92a inhibitor or siRNA-Smad7 with Lipofectamine2000 for 2 days. The cells were then harvested for measurement. Mock transfection (transfection agent without RNA) and non-targeting negative control were used as controls. The expression levels of miR-92a and Smad7 mRNA were determined using qRT-PCR, normalized to U6, and expressed as the fold change relative to the control (**$P < 0.01$ vs. control). doi:10.1371/journal.pone.0100298.g002

Figure 3. Cell injuries were determined in H9c2 cardiomyocytes. A. Lactate dehydrogenase (LDH) release. B. Malonaldehyde (MDA) release. Data are presented as mean±SEM from three independent experiments (*$P < 0.05$ and **$P < 0.01$ vs. the control group; ′$P < 0.05$ and ′′$P < 0.01$ vs. the H/R group). H/R, hypoxia/reoxygenation. doi:10.1371/journal.pone.0100298.g003
Efficiency of RNA Interference

Transfection of miR-92a inhibitor significantly decreased the level of miR-92a in cultured H9c2 cells under normoxic conditions, respectively (Figure 2A). At 50 nM, the miR-92a inhibitor significantly down-regulated miR-92a by 2.43±0.06-fold (P<0.01 vs. control). Neither mock nor NC RNA transfection affected miR-92a expression under normoxic cultures. Based on such preliminary experiments, 50 nM was chosen for subsequent experiments. At 100 nM, siRNA-Smad7 significantly decreased Smad7 expression by 3.32±0.13-fold (P<0.01 vs. control) (Figure 2B).

Inhibition of miR-92a Protects against H/R-induced Injury and Apoptosis

H/R treatment increased LDH in the culture media (16.36±0.74 vs. 8.16±0.47 ng/mL in normoxic condition, P<0.01) (Figure 3A). The miR-92a inhibitor significantly decreased LDH release in response to H/R (10.93±1.35 ng/mL, P<0.01 vs. the H/R group). Co-transfection with siRNA-Smad7 attenuated the effects of the miR-92a inhibitor.

H/R treatment increased MDA release (38.83±3.70 vs. 20.33±2.05 ng/mL in normoxic condition, P<0.01) (Figure 3B). The H/R-induced MDA release was significantly decreased by the miR-92a inhibitor (26.93±1.59 ng/mL, P<0.01 vs. the H/R group). The observed effects of the miR-92a inhibitor were also attenuated by co-transfection with siRNA-Smad7.

Figure 4. Cell death was determined in H9c2 cardiomyocytes. A. Representative dot-plot diagrams of AV/PI flow cytometry; B. Apoptotic cell percentage; C. Necrotic cell percentage. Data are presented as mean±SEM from three independent experiments (*P<0.05 and **P<0.01 vs. the control group; †P<0.05 and ‡P<0.01 vs. the H/R group). H/R, hypoxia/reoxygenation.

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**Figure 5. MiR-92a directly regulates Smad7 expression via 3’-UTR site.** A. The potential binding site for miR-92a in the 3’-UTR of Smad7 mRNA. The complementary nucleotides between miR-92a and the target region of Smad7 3’-UTR are indicated with short vertical lines. B. Luciferase reporter assay was performed by co-transfection of 293T cells with luciferase reporter containing the 3’-UTR of rat Smad7 with miR-92a mimic. Luciferase activity was determined 24 h after transfection. Data are presented as mean±SEM from three independent experiments (*P<0.05 vs. the control group).

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**Figure 6. The effect of miR-92a on SMAD7 was observed by immunocytofluorescent staining.** H9c2 cells were plated in 24-well plates and cultured to 80–90% confluence for transient transfection with the miR-92a inhibitor (50 nM) or NC (50 nM), respectively. Immunocyto-fluorescence analysis was performed 72 h after transfection. Bar: 75 μm. H/R, hypoxia/reoxygenation.

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The AV/PI dual staining (Figure 4A) revealed increased apoptosis upon H/R (27.80 ± 1.77% vs. 7.23 ± 0.40% under normoxic condition, *P < 0.01) (Figure 4B). Transfection with miR-92a inhibitor significantly decreased the percentage of apoptosis induced by H/R (18.56 ± 2.08%, *P < 0.01 vs. the H/R group). The effects of miR-92a inhibitor were attenuated by co-transfection with siRNA-Smad7.

H/R treatment also significantly increased the percentage of necrotic cells (8.56 ± 1.10% vs. 1.56 ± 0.25% in the control; P < 0.01) (Figure 4C). Transfection with miR-92a inhibitor significantly decreased the percentage of necrosis induced by H/R (6.16 ± 0.35%, **P < 0.01 vs. the H/R group). The effects of miR-92a inhibitor were also attenuated by co-transfection with siRNA-Smad7.

Smad7 is a Target of miR-92a

Bioinformatic analysis using MiRanda, miRDB, miRwalk and TargetScan suggested Smad7 as a target of miR-92a. Specifically,
In the current study, cultured H9c2 cardiomyocytes were subjected to 24-h hypoxia followed by 12-h reoxygenation. qRT-PCR analysis revealed increased expression of all miRNAs in the miR-17-92 cluster upon H/R treatment. Increased expression of miR-92a was the most prominent at 2.78-fold.

The present study showed that the inhibition of miR-92a could significantly reduce H/R-induced myocardioicte injury and apoptosis. Based on bioinformatic analyses, Smad7 was identified as a target of miR-92a. Such a prediction was confirmed by a dual luciferase reporter assay.

Through imperfect sequence-specific binding to the 3'-UTR of target miRNAs, miRNAs down-regulate gene expression by degrading target miRNAs [26,27] and/or inhibiting translation [28]. The present study demonstrated that inhibition of miR-92a significantly increased protein levels of SMAD7, but did not affect Smad7 mRNA levels, indicating that miR-92a inhibits the protein translation at the post-transcriptional level, but does not promote Smad7 mRNA degradation.

SMAD7 is an important transcriptional factor that regulates the expression of apoptosis-related genes involved in myocardial I/R injury [29,30]. SMAD7 protects against apoptosis through inhibiting the NF-kB signaling pathway [31,32]. Put together, our findings suggest that apoptosis in myocardial I/R injury, is mediated, at least partly, through the miR-92a/Smad7/NF-kB p65 pathway.

Despite of our findings, whether Smad7 is the most important target of miR-92a in cardiomyocytes (and thus the therapeutic potentials) remains unknown. Other potential candidates included (but not limited to) Pten and MKK4 [33,34]. Also, the current study did not provide direct evidence for the interaction between Smad7 and NF-kB p65. Another limitation of the current study is the use of myocardiocytes from a single species (rats), without the presence of endothelial and inflammatory cells. Based on the Hinkel et al. study that demonstrated reduced I/R-induced cell apoptosis and necrosis in HL-1 cells upon miR-92a inhibition in a murine myocyte-like cell line [20], we boldly speculate that our findings may be generalized to other species although such a generalization clearly needs to be verified.

Taken together, the current study indicated that inhibition of miR-92a can attenuate cardiomyocyte apoptosis induced by H/R via the up-regulation of SMAD7 and down-regulation of nuclear NF-kB p65.

Author Contributions
Conceived and designed the experiments: BZ MZ QZ. Performed the experiments: BZ CL JZ HL AC. Analyzed the data: BZ CL DZ ZW. Contributed reagents/materials/analysis tools: DZ ZW AC. Wrote the paper: BZ MZ CL JZ QZ.

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