Original Paper

Impact of miR-208 and its Target Gene Nemo-Like Kinase on the Protective Effect of Ginsenoside Rb1 in Hypoxia/Ischemia Injured Cardiomyocytes

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Key Words

Cardiomyocytes • Hypoxia/ischemia • Ginsenoside Rb1 • miR-208 • Nemo-like kinase • Apoptosis

Abstract

Background/Aims: Ginsenoside Rb1 (GS-Rb1) is one of the most important active pharmacological extracts of the Traditional Chinese Medicine ginseng, with extensive evidence of its cardioprotective properties. Mir-208 has been shown to act as a biomarker of acute myocardial infarction *in vivo* studies including man. However the impact of miR-208 on the protective effect of GS-Rb1 in hypoxia/ischemia injured cardiomyocytes remains unclear. The current study aims to investigate the target gene of miR-208 and the impact on the protective effect of GS-Rb1 in hypoxia/ischemia (H/I) injured cardiomyocytes. **Materials and Methods:** Primary cultures of neonatal rat cardiomyocytes (NRCMs) was subjected to the H/I conditions with or without GS-Rb1. Cell viability was calculated by MTT assay and confirmed by flow cytometry analysis. Mir-208 was then detected by qRT-PCR. Luciferase reporter assay was carried out to detect the target gene of Mir-208. Then the NRCMs were transfected with miR-208 mimics and inhibitors to evaluate the impact on cardioprotective properties of Rb1. **Results:** The miR-208 expression level was clearly upregulated in the H/I treated NRCMs accompanied by the percentage of the apoptotic cells which could be reversed by GS-Rb1 pretreatment. The nemo-like kinase (NLK) mRNA and protein expression levels were decreased in H/I group measured by RT-PCR and western blotting. Luciferase activity assay was then carried out to identify that NLK may be a direct target of mir-208. MTT assay showed that miR-208 inhibitor slightly decreased the protective effect of Rb1 on the H/I impaired

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NRCMs. However, results showed no statistical difference. **Conclusions:** These findings proved that NLK was a direct target of miR-208 and miR-208 act indirectly during Rb1 protecting H/I impaired NRCMs and further researches were needed to explore the relationship that microRNAs and other signal pathways in the protective effect of GS-Rb1 on the hypoxia/ischemia injuries in cardiomyocytes.

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Introduction

Myocardial infarction (MI), the one of the leading causes of death throughout the world, presents a major challenge to the health services because cases contribute significantly to healthcare costs and the degree of disability among the adult population. There is now evidence that microRNAs (miRNAs) are involved in the causes, processes, development and consequences of MI, making it possible that they can act a target for the drug design [1-4].

Nemo-like kinase (NLK), a Wnt/ β -catenin signaling inhibitor, regulates diverse signaling processes by phosphorylating several transcription factors [5]. Activation of Wnt/ β -catenin signaling pathway could directly induce apoptosis in many mammalian cells. In cardiomyocytes, Wnt/ β -catenin signaling has a multifunctional role in heart cell generation, proliferation and differentiation [6, 7]. Further evidence suggests that Wnt/ β -catenin signaling is involved in heart disease as well as myocardial infarction [8-11].

MicroRNAs (miRNAs) have emerged as a novel class of endogenous, small (<24 nucleotide), noncoding RNAs with strong biological functions in the cardiovascular system [12, 13]. Mature miRs regulate the repression of specific target gene translation and/or promote the degradation of their transcribed mRNAs by binding to the 3'-untranslated regions (UTR) of the target genes [14, 15]. miRNAs provide an additional post-transcriptional layer of spatial and temporal control of developmental and homeostatic events by altering levels of critical regulators within complex genetic pathways [16-18]. As a group, it is estimated that miRNAs are estimated regulate 30% of the genes in the human genome [19].

Further evidence implicates miRNA function as integral in regulatory cellular functioning, including proliferation, apoptosis, and many physiological pathways. MiR-208 is specifically expressed in the mammalian cardiomyocytes, being encoded by an intron of the α -cardiac muscle myosin heavy chain gene (Myh6) and required for cardiomyocyte hypertrophy and fibrosis [20]. miR-208 sequences in humans, mice, rats, and dogs are identical, with pre-miRNA being highly conserved in mammals. In acute myocardial infarction in patients and animals, miR-208 is markedly increased in plasma [21-23] and even in urine [24], indicating that it could be a useful diagnostic biomarkers. However, the involvement of miR-208 in hypoxic- and ischemic-induced cardiomyocyte apoptosis is unclear.

The root of *Panax ginseng* is one of the most popular herbal medicines for the treatment of many ailments, particularly those associated with aging and cardiac deterioration, which has been used for several thousand years in China [25, 26]. Ginsenoside Rb1, one type of protopanaxadiol saponin, is one of the most important active compounds of ginseng [27], and an increasing amount of evidence has indicated that Rb1 possesses cardioprotective effects both *in vitro* and *in vivo* [28]. However, the target miRNA of Rb1 and the related mechanism has not been fully characterized. In the present study, we found that miR-208 expression was upregulated in the cardiomyocytes *in vitro* and accompanied with cell apoptosis induced by hypoxia/ischemia treatment, which could be ameliorated by Rb1 pretreatment. Additionally, the target gene of miR-208 was identified by luciferase reporter assay. Then, miR-208 mimics and miR-208 inhibitor was transfected to the neonatal rat cardiomyocytes to investigate the impact on the cardioprotective effect of Rb1 on the cardiomyocytes impaired by hypoxia/ischemia treatment.

Materials and Methods

Materials

GS-Rb1 (catalog number #110704), purchased from National Institutes for food and drug Control, was dissolved in phosphate-buffered saline (PBS) to create a stock solution for subsequent dilution. Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco (Grand Island, NY, USA). DMSO and 3-(4, 5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) were obtained from Sigma (St. Louis, MO, USA). Annexin V-FITC/PI kit was purchased from Kaiji Biological Engineering Institute. Cell Lysis Buffer for Western blotting, and IP and Enhanced BCA Protein Assay Kit were obtained from Beyotime (Haimen, China). The primary antibody against NLK (catalog number # Ab97642) was purchased from Abcam. Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG secondary antibody (catalog number # A0545) was obtained from Sigma. Enhanced chemiluminescence kit was obtained from Millipore (Billerica, MA, USA). miRNA extracting kit (CW0627) was purchased from Beijing Cowin Biotech Co., Ltd.

Culture of neonatal rat cardiomyocytes and transfection (NRCMs)

Primary cultures of NRCMs from 12-24 h old Sprague Dawley rats (Vital River Laboratories, Beijing, China) was prepared by means of gentle serial trypsinization as previously described [29]. All experiments were approved by the Beijing Ethics Committee for the Use of Experimental Animals. The NRCMs were plated in collagen-coated 96- or 6-well plates and maintained at 37°C in a 5% CO₂/95% air humidified incubator in DMEM containing 10% (v/v) fetal bovine serum, 100 U/mL penicillin and 100 mg/mL streptomycin. The following experiments used spontaneously beating cardiomyocytes 48-72 h after plating.

The miRNA mimics and inhibitors were synthesized by Hanbio Biotechnology Co.Ltd. (Shanghai, China) and were transfected into cells using Lipofect-amine 2000 (Invitrogen, Carlsbad, CA, USA). Total RNA and protein were prepared at 48 h after transfection for qPCR and Western blot analysis, respectively.

Hypoxia/ ischemia treatment

For the H/I protocol, hypoxia was induced with the MGC AnaeroPack System in a AnaeroPack jar (Mitsubishi Gas Chemical Co., Tokyo, Japan), depleting the concentration of O₂ to 10 % in 2h. Ischemia was reached by replacing culture medium with DMEM without glucose (Gibco, Grand Island, USA) and serum. NRCMs with or without GS-Rb1 were placed in the AnaeroPack jar and then put into a 37°C incubator for 24 h. Control plates were kept under normoxic conditions for the corresponding time.

MTT assay

NRCMs viability was determined by the MTT assay. Cardiomyocytes were plated on 96-well dishes at 2×10^4 cells/well. MTT (5 mg/mL) was added to each well immediately after hypoxia/ ischemia treatment. Plates were incubated for 4 h at 37°C, the medium aspirated from each well and 100 µL of DMSO added to dissolve the formazan crystals. The optical density of each well was read at 492 nm using a Microplate Reader (Bio-Rad, Hercules, CA). Results are given as percentages of the control group (100%).

Apoptosis assay by Annexin V/PI staining

NRCMs were harvested and washed with PBS at the indicated time-points. The percentage of normal (non-apoptotic) and apoptotic cells was measured by double supravital staining with Annexin-V and PI, using an Annexin V-FITC Apoptosis Detection kit (KeyGen, Nanjing, China). Flow cytometric analysis used a Cytomics FC500 flow cytometer with CXP software (Beckman Coulter, Fullerton, USA), the operator being blind of the group assignment.

Western Blotting.

NRCMs under different hypoxic and ischemic conditions were harvested and lysed with Cell Lysis Buffer for Western blotting and IP. Protein concentration was measured using a BCA Protein Assay Kit. Equal amounts of sample lysate were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred by electroblotting to a nitrocellulose membrane (Millipore). The membrane was blocked with 5% non- fat milk in TBST buffer (20m M Tris-HCl, pH7.4, 150 mM NaCl and 0.1% Tween 20) overnight at 4°C. The membrane was incubated with specific primary antibodies for 2 h and an IgG HRP conjugated specific secondary antibody for 1 h at room temperature. The signals were visualized

Table 1. Primer pairs used for qRT-PCR

Primers	Sequences 5'-3'
Mir-208 RT primer	CTCAACTGGTGTCTGAGTCGGCAATTCAGTTGAGGCTTTTG
Mir-208 Forward primer	ACACTCCAGCTGGGATAAGACGAGCA
Mir-208 Reverse primer	TGGTGTCTGAGTCTG
U6 Forward primer	CTCGCTTCGGCAGCACA
U6 Reverse primer	AACGCTTCACGAATTTGCGT
NLK Forward primer	CTAGATGAAGGGCGGCTGAG
NLK Reverse primer	GAGCGACGGTGAAGTATA
GAPDH Forward primer	CACCATCTTCCAGGAGCGAG
GAPDH Reverse primer	AAATGAGCCCCAGCCTTCTC

by enhanced chemiluminescence (ECL) (Pierce) on a Syngene G: BOX Chemi gel documentation system (Syngene, Cambridge, UK). Densitometric values were normalized in each group using GAPDH as an internal control.

RNA extraction and quantitative reverse transcription- polymerase chain reaction (qRT-PCR)

Rno-mir-208a (MIMAT0000880) mature sequence was: AUAAGACGAGCAAAAAGC. Total RNA from cells in control, H/I treated, mir-208 mimic group or the negative control miRNA was isolated using the RNA extracting kit (Covin Biotech) following the manufacturer's instructions.

Primer pairs used for qRT-PCR are given in Table 1. cDNA synthesis was done with 1 µg total RNA using an RT-PCR reagent kit (TOYOBO), and cDNA quantified by PCR using the SYBR Green Master Mix (Roche). The PCR program was 95°C for 5 min followed by 40 cycles of 95°C for 20 s, 60°C for 30 s, and 72°C for 20 s. The relative expression level of NLK was normalized to that of GAPDH by the $2^{-\Delta\Delta C_t}$ cycle threshold method. The relative fold-change in mir-208 expression level was calculated to that of U6 by the $2^{-\Delta\Delta C_t}$ cycle threshold method.

Luciferase reporter assay

miR-208 target sequences were inserted between the XhoI and NotI restriction sites in the 3' UTR of the hRluc gene in the psiCHECK-2 vector (Promega, Madison, WI, USA). Fragments of NLK 3'UTR were amplified from the genomic DNA using the forward primer: 5'-ACACTCGAGAAGTTCAATGTGGAGACTTGAAAC-3' and the reverse primer: 5'-ATTAGCGGCCGCCATTGAAGTTAAACTTCC-3'. Cells were cotransfected with 200 ng psiCHECK2 constructs and 30 nM miR-208 mimics or miR-negative control in 24-well plates. One day after transfection, firefly and Renilla luciferase activities were measured by the Dual-Glo Luciferase assay system (Promega). Normalized data were calculated as the quotient of Renilla/firefly luciferase activities. Three independent experiments were run in triplicate.

Construction of plasmids and transfection

For lentivirus-mediated overexpression of NLK, the NLK cDNA was inserted into a pLVX-Zs Green expression vector. Recombinant lentiviruses expressing NLK was produced by Genelive (Shanghai, China). The NRCMs cells were infected with concentrated virus according to the manufacturer's instructions. The NLK expression in the infected cells was validated by western blotting assay.

Statistical analysis

Data are given as means $\pm$ SD, and Student's t-test was used for comparisons. $p < 0.05$ was taken as a significant difference between groups.

Results

H/I Induced Cardiomyocytes apoptosis and cardioprotective effect of Rb1

Effect of H/I induced NRCMs apoptosis was examined by the MTT assay and confirmed by Annexin V-FITC/propidium iodide (PI) double-staining followed by flow cytometry analysis (Fig. 1A). Compared with the control group, H/I treatment from 3 to 24 h decreased NRCMs

viability from $77.7 \pm 4.2\%$ to $25.7 \pm 3.2\%$. With the presence of Rb1 ($40 \mu\text{M}$), cell viability increased for more than 2 times compared to the H/I 24 h group. To examine the effect of H/I treatment on the cell death, flow cytometry analysis was performed and the results showed that the percentage of living cells after 24 h H/I treatment markedly decreased in H/I group compared to the control group, and could be reduced by GS-Rb1 treatment (Fig. 1 B-D, B1 box).

NLK is a direct target of miR-208

The level of miR-208 was detected by real-time RT-PCR assay. The expression level of miR-208 in H/I group increased nearly 10 folds versus control, with the treatment of GS-Rb1, the expression change of miR-208 could be reversed.

Since we have already shown that H/I treatment markedly unregulated mir-208 expression in NRCMs, to validate whether miR-208 regulates NLK directly through a putative binding site in NRCMs, we cloned NLK 3'-UTR in the predicted miRNA binding site into the luciferase gene (pSiCHECK2; Promega). Following cotransfection with the pSiCHECK2 vectors and miR-208 or the negative control miRNA, the upregulation of miR-208 in NRCMs transfected with miR-208 significantly decrease luciferase activity of the wild-type NLK 3'-UTR (Fig. 2; $P < 0.05$), clearly indicating that NLK is a direct target of miR-208.

miR-208 negatively regulates NLK expression in NRCMs

To investigate the correlation between miR-208 and NLK, miR-208 was upregulated in NRCMs by treatment with miR-208 for 48 h. Expression of NLK mRNA and protein was measured by qRT-PCR and western blot analysis. NLK mRNA expression in cells transfected with the negative control vector was 6-fold higher than that in the cells transfected with miR-208. Western blot analysis showed that upregulated miR-208 leads to a corresponding decrease in endogenous NLK protein (Fig. 3), suggesting that miR-208 negatively regulates NLK expression.

Fig. 1. Effects of Rb1 on the hypoxia/ ischemia-induced NRCMs death. NRCMs with or without Rb1 were incubated with normal or H/I conditions from 3 h to 24 h. A. Cell viability was determined by MTT assay. B-D. NRCMs in control, H/I 24 h and H/I 24 h + Rb1 group was stained with Annexin V-FITC/PI and analyzed by flow cytometry. ** $p < 0.01$, *** $p < 0.001$ versus control, ## $p < 0.01$ versus H/I 24 h group. (n = 3).

Fig. 2. Identification of target sites in the 3' UTR of rat NLK. A. qRT-PCR analysis of miR-208 in NRCMs. B. Construct map of psiCHECK-2/ NLK used for the luciferase assay. The 3'UTR of NLK was cloned into the vector after the luciferase gene to form a fusion transcript. C. Schematic diagram of miR-208 binding sites in the 3' UTR. D. Relative renilla luciferase activity of psiCHECK-2/ NLK in the presence of miR-208 or miR-neg expression vectors in NRCMs, and normalized to firefly luciferase activity. ***P < 0.01 compared with those transfected with miR-neg expression vectors.

Fig. 3. NLK protein expression was inhibited by the upregulation of miR-208 in NRCMs cells. (A) The real time PCR shows that the NLK gene was suppressed by the over expression of miR-208. (B) A representation of western blotting showing the expression of NLK protein was suppressed by the upregulation of miR-208. ***P<0.001 versus negative control group. This indicates that miR-208 inhibited NLK protein expression in NRCMs cells.

Fig. 4. Impact of miR-208 on the cardioprotective effect of Rb1. (A) NRCMs was transfected with miR-208 blank, mimics and inhibitors. Phase contrast light microscope fluorescence images of the cells were taken At 48 h after transfection. (B) MTT assay was performed to evaluated cell viability of each group.

Impact of miR-208 on the cardioprotective effect of Rb1

In order to verify the impact of miR-208 on the cardioprotective effect of Rb1, miR-208 blank, mimics and inhibitors were transfected into NRCMs (Fig. 4A). All the cells with or without Rb1 were subjected to 24 h H/I treatment. Cell viability was calculated by MTT assay. MiR-208 blank, mimics and inhibitors had no effect on the NRCMs viability, as well as response to the H/I injuries (Fig. 4B). But miR-208 inhibitor slightly decreased the protective

Fig. 5. Impact of NLK on the cardioprotective effect of Rb1. (A) A representation and analysis data of western blotting showing the lentivirus-mediated overexpression of NLK in NRCMs with or without H/I injury. (B) MTT assay was performed to evaluated cell viability of each group. *P<0.05 versus control group; #P<0.05 versus H/I group.

effect of Rb1 on the H/I impaired NRCMs. However, results showed no statistical difference.

Impact of NLK on the cardioprotective effect of Rb1

Since overexpression of miR-208 had no effect on the NRCMs viability, and given that NLK is a direct target of miR-208, we hypothesized that NLK may neither be involved in the cardioprotective effect of Rb1. To investigate this, we assessed the effect of NLK overexpression on NRCMs cell growth by MTT assay. Treatment of NRCMs with H/I injury suppressed the expression of NLK (Fig. 5A), while GS-Rb1 treatment significantly preserved the expression of NLK. Furthermore, the exogenous overexpression of NLK did not affect the NRCMs viability with or without H/R injury. (Fig. 5B). This suggests that downregulation of NLK expression by miR-208 did not involved in the cardioprotective effect of Rb1 against H/I injury.

Discussion

Mir-208, strictly expressed in cardiomyocytes, act as a biomarker for MI diagnosis. Therapeutic inhibition of miR-208a by subcutaneous treatment with anti-miR-208a during hypertension-induced heart failure in Dahl hypertensive rats prevented pathological myosin switching and cardiac remodeling, while improving cardiac functioning, overall health and survival [30]. With other target genes of mir-208, it has been proved that miR-208 was necessary for the production of alpha-myosin heavy chain and controlling the response of the heart to hypertrophic stimuli [20]. MiR-208 was also involved in insulin-induced vascular smooth muscle cell proliferation by downregulating p21, a key member of CDK-inhibitory protein family [31]. In preosteoblasts, miR-208 is closely involved in preosteoblast differentiation by partially regulating its target gene, V-ets erythroblastosis virus E26 oncogene homolog 1 (Ets1) [32]. However, we provide new that NLK was the target gene of mir-208 with regard to cardiomyocyte apoptosis.

The NLK gene could be related to diabetic hearts, like the mir-208 putative target gene [33]. This prediction of the NLK gene involvement was examined using TargetScan. human6.2. Western blotting was also showed that NLK was downregulated in the H/I group, whereas mir-208 was dramatically increased. Furthermore, to identify whether miR-208 can genuinely regulate the expression of NLK, NLK 3'-UTR was cloned into the pSiCHECK2, giving the 3'-UTR the majority of potential miRNA binding sites downstream of the coding sequence for luciferase (pMiR-Report; Promega). Cardiomyocytes were cotransfected with the pSiCHECK2 vector containing the 3'-UTR and miR-208 or negative miRNA. Overexpression of miR-208 significantly reduced luciferase activity from the reporter construct containing

the NLK 3'-UTR, suggesting that NLK is a direct miR-208 target. Consider the fact that exogenous overexpression of NLK did not affect the NRCMs viability with or without H/R injury, down-regulation of NLK expression by miR-208 is not involved in the cardioprotective effect of Rb1 against H/I injury.

However, we only get the negative results when transfected miR-208 mimics and inhibitors into the NRCMs to prove the impact of miR-208 on cell viability and the cardioprotective effect of Rb1. We speculated that, miR-208 act indirectly during Rb1 protecting H/I impaired NRCMs. We have proved that, several microRNAs involved in cardioprotective properties of Rb1 [34] and H/I induced cardiomyocytes apoptosis could be regulated by the mitochondria apoptotic pathway [29]. This leaves open the possibility of exploring the relationship of miR-208 with the mitochondrial apoptotic pathway in H/I induced cardiomyocyte apoptosis.

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Disclosure Statement

The authors declare that they have no conflict of interests.

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