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Original Article

Downregulation of microRNA-100 protects H_2O_2-induced apoptosis in neonatal cardiomyocytes

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Abstract: Hypoxia or reoxygenation-induced cardiomyocyte apoptosis is one of the major causes of cardiac dysfunction. Recently, regulations of microRNAs were shown to play important roles in cardiomyocyte apoptosis. MicroRNA-100 (miR-100) is one of the cardiac miRNA that was up-regulated in failing heart. In this study, we identified that miR-100 expression was up-regulated in H_2O_2-induced apoptosis in neonatal mice cardiomyocytes in a time-dependent manner. Furthermore, functional analysis revealed that miR-100 downregulation attenuated H_2O_2-induced apoptosis. Through biochemical analysis of western blot, we found that miR-100 suppressed the expression of insulin-like growth factor 1 receptor (IGF1R) during the process of hypoxia-induced apoptosis in cardiomyocytes. More importantly, ectopic down-regulation of IGF1R reversed the protective effect of miR-100 down-regulation on H_2O_2-induced apoptosis, revealing that miR-100 regulates cardiomyocyte apoptosis through the association of IGF1R. Taken together, our data demonstrated the functional role miR-100 in H_2O_2-induced apoptosis in cardiac dysfunctions.

Keywords: Cardiomyocytes, apoptosis, microRNA-100, IGIFR

Introduction

Heart attack or acute myocardial infarction (AMI) is the major cause of morbidity and mortality in the world [1]. AMI may lead to myocardial ischemia or reperfusion (I/R) injury due to increased permeability of capillaries and arterioles [2]. Though great progress had been made on understanding the pathophysiologic mechanisms of myocardial ischemia or reperfusion injury, the complete profile of molecular pathways associated with myocardial ischemia is largely unknown [3].

MicroRNAs (miRNAs) are groups of noncoding short-arm (18~22 BP) RNAs that regulate various biological processes by suppressing the translation or degrading the transcription of targeted genes [4]. Many of the miRNAs are abundantly expressed in mammalian heart and play critical role in regulating myocardial ischemia [5]. Among those identified heart-associated functional miRNA, microRNA-100 (miR-100) was shown to be up-regulated in failing heart through the regulation on beta-adrenergic receptor-mediated cardiac genes [6].

In the present study, we cultured cardiomyocytes from P1 C57BL/6 mice *in vitro*. Then we applied H_2O_2 to induce hypoxia-like cardiac injury, as oxygen-related free radicals generated by hypoxia contributed significantly to myocardial ischemia [7]. Then, the gene expression profile of miR-100 was examined to see whether it was directly affected by H_2O_2-induced cardiomyocyte cytotoxicity or apoptosis. Furthermore, we ectopically down-regulated miR-100 in H_2O_2 injured cardiomyocytes to see if inhibiting miR-100 may protect apoptosis and promote survival of cardiomyocytes in response to H_2O_2-induced injury. Finally, we assessed the associated signaling pathways which could be directly involved in the regulation of miR-100 on cardiomyocyte apoptosis. The data of our study would undoubtedly broaden our understanding on the molecular mechanism of miR-100 on regulating myocardial diseases, and help us seek optimal

clinical methods to treat patients with myocardial ischemia.

Material and methods

Primary cardiomyocyte culture and H_2O_2 treatment

Cardiomyocytes were extracted from P1 C57BL/6 mice according to a previously described method [8]. Briefly, pups were sacrificed and heart was quickly immersed in ice-cold Hanks Balanced salts solution (HBSS, Invitrogen, USA). Atria were removed and the remaining ventricular tissues were treated with 1 mg/ml collagenase II in warm (20 C) HBSS for 20 minutes. Cell suspensions were then collected by centrifuging and re-suspension in DMEM/F12 medium (Gibco, USA) supplemented with 5% fetal bovine serum (FBS, Invitrogen, USA), 0.5 mM L-Glutamine (Invitrogen, USA), and 1% streptomycin/penicillin (Sigma Aldrich, USA). The cells were then maintained in 6-well tissue-culture plates at 37 C in DMEM/F12 + 2% FBS, 0.5 mM L-Glutamine (Invitrogen, USA), and 1% streptomycin/penicillin (Sigma Aldrich, USA), supplied with 5% CO_2. To induce hypoxia-like apoptosis, 100 μM H_2O_2 was added into cardiomyocyte culture for 6 h, 12 h, 24 h or 27 h.

Cell viability assay

Cardiomyocytes were plated in 96-well plates. Cell viability was assessed using a MTT assay. After H_2O_2 treatment, 10% MTT (Sigma Aldrich, USA) was added to the culture medium of DMEM/F12 + 2% FBS for 4 hours. The MTT-formazan crystals were diluted in DMSO and the absorption at 570 nm was evaluated with a Synergy HT multi-detection microplate reader (Bio-Rad, USA). The percentages of viable cardiomyocytes with H_2O_2 treatment were estimated against the percentage under control (0 h H_2O_2 treatment).

RNA isolation, reverse transcription, and quantitative real-time PCR (qRT-PCR)

Cardiomyocytes were detached from 6-well plates with 0.05% trypsin-EDTA (Gibco, USA) and pooled. Total RNA was isolated using Trizol reagent (Invitrogen, USA), treated with DNAseI (Invitrogen, USA), and reverse transcribed using a NCode miRNA First-Strand cDNA Synthesis kit (Invitrogen, USA). Quantitative real-time PCR was conducted by an iCycler (Biorad, USA) with the iQ SYBR Green Super Mix. Expression levels of miR-100 were normalized to endogenous U6 snRNA and presented as folder changes ($2^{-\Delta\Delta Ct}$) against control [9].

Downregulation of miR-100

MicroRNA-100 inhibitor (miR-100-Inhibitor) was synthesized by RiboBio (RiboBio, China). In cardiomyocyte culture, cells were transfected with 200 nM miR-100-Inhibitor by Lipofectamine 2000 24 hours before H_2O_2 treatment. In control experiments, cardiomyocytes were transfected with 200 nM non-specific miRNA (miR-NC, RiboBio, China).

TUNEL assay

The apoptosis of cardiomyocytes was examined by a TUNEL assay. Briefly, cultured cardiomyocytes were fixed with 4% paraformaldehyde (PFA) (Millipore, USA) and permeabilized with 1% Triton X-100 (Sigma Aldrich, USA) and in phosphate-buffered saline (PBS) (Invitrogen, USA) for 30 minutes, followed by 3 times (3 × 10 mins) wash of fresh PBS. Then, an Apo-BrdU in Situ DNA Fragmentation Assay Kit (BioVision, USA) was applied for 1 hour, followed by incubating the treated plates with 5 μl anti-BrdU-FITC antibody. Fifteen minutes of DAPI immunostaining was conducted to identify the nuclei of cardiomyocytes. Images were then taken on an inverted Leica TCS-SP2 AOBS confocal laser-scanning microscope (Leica, Germany). Apoptosis was quantified as the percentage of healthy (no apoptosis) cardiomyocytes, and normalized to the percentage under control condition.

Western blot analysis

Resuspended cardiomyocytes were lysed by a lysis buffer (20 mM Hepes, pH7.9, 350 mM NaCl, 20% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 1% NP-40, 1 mM orthovanadate, 0.1 mM DTT, 25 mM NaF, and protease inhibitor cocktail; Sigma Aldrich, USA). Twenty microgram of total protein was resolved on a 10% SDS-PAGE gel and transferred to a PVDF membrane. Incubation of primary antibody against insulin-like growth factor 1 receptor (IGF1R) (1:200, Cell Signaling, USA) was applied overnight at 4°C. On second-day, respective horseradish peroxidase-coupled secondary antibody (Cell Signaling, USA) was applied for 1 hour at RT. The blot was visualized with Pierce ECL reagents and GAPDH was the control protein.

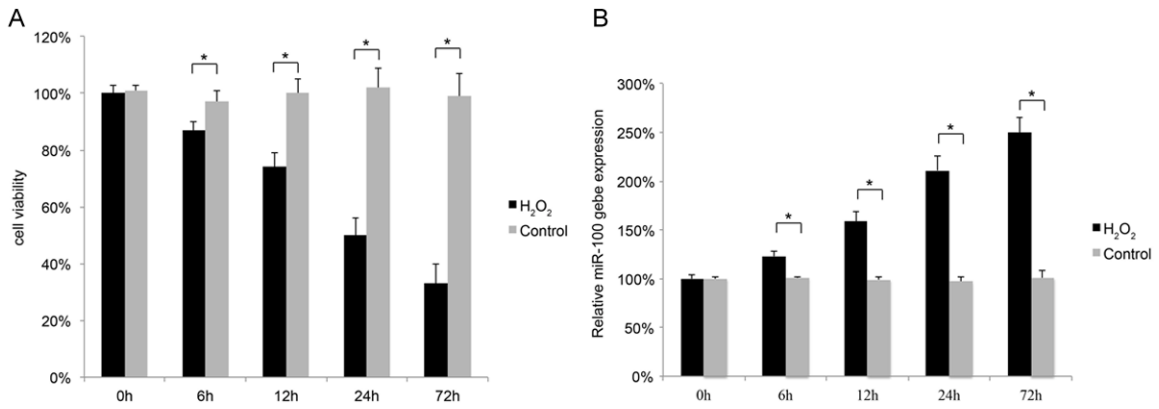

Figure 1. The effect of H_2O_2 on cytotoxicity and miR-100 expression in cardiomyocytes. Cardiomyocytes were extracted from P1 C57BL/6 mice and cultured in vitro. They were treated with DMEM + 5% FBS (control), or additional 100 μM H_2O_2 for 0, 6, 12, 24 and 72 hours. A. The H_2O_2-induced cytotoxicity was estimated by a cell viability assay (*P<0.05). B. The gene expression levels of miR-100 were measured by qRT-PCR (*P<0.05).

IGF1R down-regulation assay

IGF1R siRNA (RiboBio, China) was used to knock down IGF1R gene in cardiomyocytes. A non-specific siRNA (NC_siRNA, RiboBio, China) was also used in the experiment. The transfection of siRNAs (1 μM) was performed by a Lipofectamine 2000 reagent per manufacturer's protocol.

Lactate dehydrogenase (LDH) assay

A cytotoxicity detection kit (Roche, USA) was used to determine the amount of released LDH. A microplate luminometer (Turner Biosystems, USA) was used to measure the absorbance at 490 nm, and the percentage of cytotoxicity was then determining per manufacturer's protocol.

Statistical analysis

All data were shown as mean ± S.E.M. Statistical analysis was performed by a GraphPad Prism software (version 3.0). For comparison, a student's t-test was conducted to determine statistical significance (*P<0.05). All experiments were at least repeated three times.

Results

H_2O_2 induced cytotoxicity and upregulated miR-100 in cardiomyocytes in time-dependent manner

We first examined the effect of H_2O_2-induced cytotoxicity in cardiomyocytes. We cultured mouse primary cardiomyocytes *in vitro* and treated them with various durations of 100 μM H_2O_2 to induce hypoxia-like cytotoxicity. At 6, 12, 24 and 72 hours after H_2O_2 treatment, the cytotoxicity was measured by a cell viability assay. The results showed that 6, 12, 24 or 72 hours treatment of 100 μM H_2O_2 induced significant cell deaths among cardiomyocytes with increased cytotoxicity in a time-dependent manner (**Figure 1A**, *P<0.05). We also examined the gene expression levels of miR-100 in response to H_2O_2 treatment. The results demonstrated miR-100 was significantly upregulated by 6, 12, 24 or 72 hours treatment of 100 μM H_2O_2, also in a time-dependent manner (**Figure 1B**, *P<0.05).

Inhibition of miR-100 reduced H_2O_2-induced cardiomyocyte apoptosis

Since we found miR-100 was upregulated by H_2O_2 in cardiomyocytes, we suspected miR-100 might play a critical role in cardiomyocytes apoptosis regulation. To test this hypothesis, we transected cardiomyocytes with 200 nM miR-100-inhibitor to ectopically down-regulate miR-100 in cardiomyocytes. Twenty-four hours after transfection, those cardiomyocytes were treated with 100 μM H_2O_2 for additional 24 hours, followed by a TUNEL immunostaining assay to examine the effect of miR-100 down-regulation on H_2O_2-induced cardiomyocyte apoptosis. To verify the specificity of miR-100-inhibitor, a non-specific miRNA (miR-NC) was also used in the transfection in cardiomyocytes. The results demonstrated that while H_2O_2 induced significant apoptosis in cardiomyocytes as more TUNEL-positive cells were

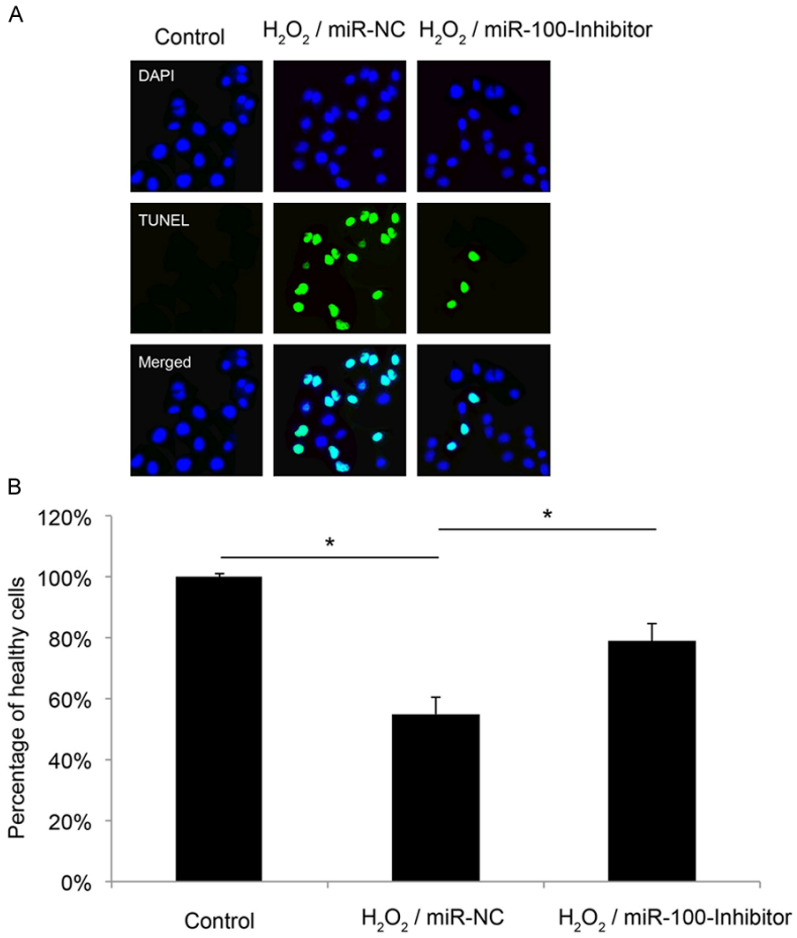

Figure 2. MiR-100 downregulation protected cardiomyocyte from H_2O_2-induced apoptosis. A. Cardiomyocytes were either untreated (control), or treated with 100 μM H_2O_2 for 24 hours. For the cardiomyocytes treated with H_2O_2, some were pre-transfected with 200 nM miR-100-inhibitor for 24 hours, and the others were pre-transfected with 200 nM miR-NC. TUNEL assay was performed and the representative immunofluorescent images were shown for apoptotic (TUNEL-positive) cardiomyocytes. DAPI staining was also performed. B. Percentages of healthy (TUNEL-negative) cardiomyocytes were evaluated for cardiomyocytes without any treatments (Control), treated with H_2O_2 plus miR-NC, or treated with H_2O_2 plus miR-100-Inhibitor (*P<0.05).

observed (**Figure 2A**, H_2O_2/miR-NC vs. Control), down-regulation of miR-100 significantly reduced the TUNEL-positive cardiomyocytes (**Figure 2A**, H_2O_2/miR-100-Inhibitor vs. H_2O_2/miR-NC). Further measurement on the percentage of healthy (TUNEL-negative) cardiomyocytes verified the protective effect of down-regulating miR-100 on H_2O_2-induced apoptosis (**Figure 2B**, *P<0.05).

IGF1R directly mediated the protection of miR-100 downregulation on cytotoxicity in cardiomyocytes

Finally, we wondered what were the downstreaming signaling pathways involved in miR-100 regulation on H_2O_2-induced cytotoxicity in cardiomyocytes. Western blotting analysis demonstrated that IGF1R was upregulated while miR-100 was down-regulated during H_2O_2-induced apoptosis (**Figure 3A**). Thus, we hypothesized that IGF1R may directly mediate the protection of miR-100 downregulation on H_2O_2-induced cytotoxicity in cardiomyocytes. To examine that, we conducted the experiment of double-transfections on cardiomyocytes before H_2O_2 treatment. First, cardiomyocytes were transfected with 200 nM miR-100-inhibitor for 24 hours to down-regulate miR-100. Second, cardiomyocytes were treated with 100 μM H_2O_2 for another 24 hours. Third, 12 hours before H_2O_2 treatment, cardiomyocytes were either transfected with IGF1R_siRNA to down-regulate IGF1R, or NC_siRNA. The resulted effect on cardiomyocyte cytotoxicity, assessed by LDH assay, demonstrated that significantly higher percentage of cardiomyocytes had cell deaths while IGF1R was down-regulated (**Figure 3B**, *P<0.05). Thus, our results strongly suggested that IGF1R was directly involved in the protection of miR-100 down-regulation on H_2O_2-induced cytotoxicity in cardiomyocytes.

Discussion

In the present study, we demonstrated that cultured mammalian cardiomyocytes were significantly injured by 100 μM H_2O_2 as early as 6-hour treatment. This result is in line with previous studies showing hypoxia conditions generated oxygen-derived free radicals in cardiac tissues, thus played pivotal role in generating myocardial ischemia [7].

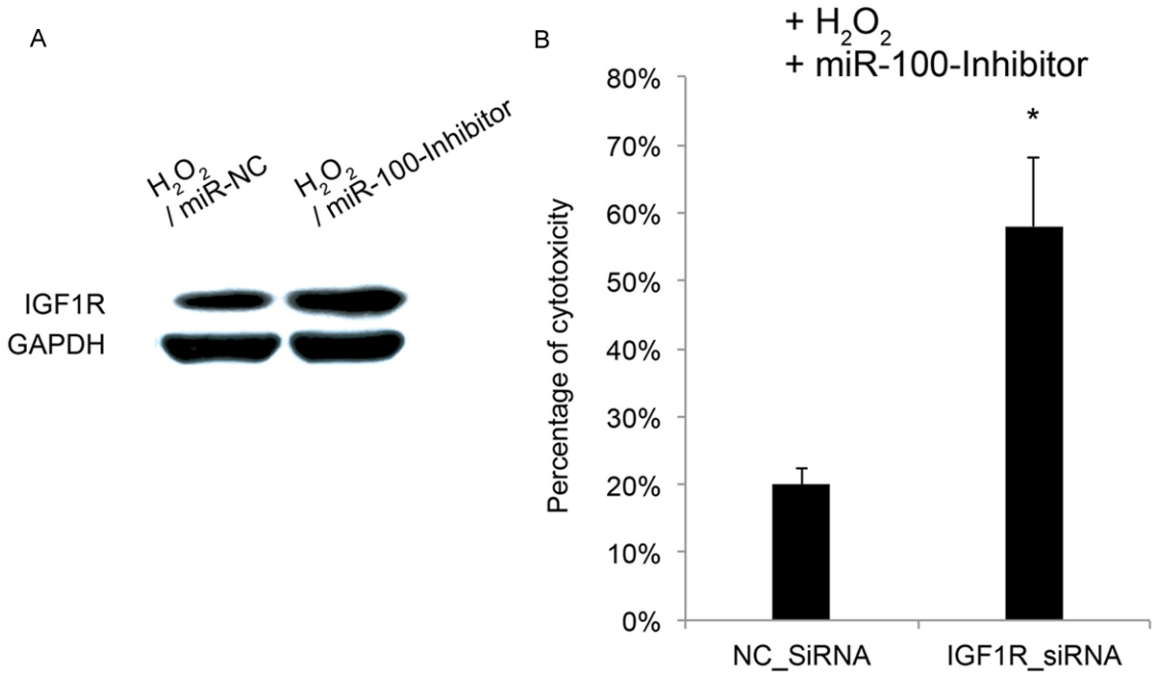

Figure 3. IGF1R was involved in miR-100 mediated apoptosis protection in cardiomyocytes. A. Cardiomyocytes were transfected with either miR-NC or miR-100-inhibitor for 24 hours, followed by another 24 hours of H_2O_2 treatment. Western blot was applied to examine the protein expressions of IGF1R. GAPDH was the internal control. B. Cardiomyocytes were transfected with miR-100-inhibitor for 24 hours, followed by another 24 hours of H_2O_2 treatment. Twelve hours before H_2O_2 treatment, cardiomyocytes were transfected with either NC_siRNA or IGF1R_siRNA. Twenty-four hours after H_2O_2 treatment, LDH essay was used to determine the level of toxicity in cardiomyocytes based on measured optical density (*P<0.05).

Also in the present study, we demonstrated that miR-100 was upregulated by H_2O_2-induced cytotoxicity in cultured neonatal mammalian cardiomyocytes. MicroRNAs have long been implied in regulating ischemia injury in heart [5], and miR-100 was shown to be up-regulated in failing heart [6]. The results of our study showing that miR-100 was also up-regulated along with the pathological condition of H_2O_2-induced cytotoxicity in neonatal cardiomyocytes, along with previous reports, suggest that miR-100 was normally to be lowly expressed in both neonatal and adult cardiac tissues, and upregulation of miR-100 was the unanimous response to pathological cardiac conditions throughout the development stages of heart.

In previous study, it was shown that upregulation of miR-100 in failing heart contributed to the upregulation of adult gene of sarcoplasmic reticulum Ca^{2+} ATPase (SERCA2a), as well as the upregulation of the fetal genes ANF and βMyHC [6]. In the present study, we revealed that IGF1R was likely the other target of miR-100 in apoptosis regulation in cardiomyocyte.

We showed that during the protective process of miR-100 down-regulation on H_2O_2-induced cardiac cytotoxicity, IGF1R was subsequently upregulated. Moreover, we demonstrated that down-regulation of IGF1R ameliorated the protective effect of miR-100 down-regulation on H_2O_2-induced cardiomyocyte cytotoxicity. IGF1R was shown to be abundantly expressed in various types of heart tissues [10, 11], and play important role in regulating heart size during development [12]. However, no direct evidence has suggested that IGF1R was directly associated with miR-100 regulation in pathological cardiac conditions, until this study. Our results revealing new mechanism of IGF1R in miRNA regulation during the process of hypoxia-induced cytotoxicity might help identify new molecular target involved in the process of myocardial ischemia.

Overall, our data presented new molecular mechanism of miR-100, which is its down-regulation protected cardiomyocytes against H_2O_2-induced cardiac cytotoxicity and apoptosis. Our work may provide new clinical strategy to treat

patients with conditions of acute myocardial infarction.

Disclosure of conflict of interest

None.

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