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 downregulation 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, IGF1R

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$_2$O$_2$ 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) supplement-ed with 5% fetal bovine serum (FBS, Invitrogen, USA), 0.5 mM L-Glutamine (Invitrogen, USA), and 1% streptomyacin/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% streptomyacin/penicillin (Sigma Aldrich, USA), supplied with 5% CO$_2$. To induce hypoxia-like apoptosis, 100 μM H$_2$O$_2$ 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$_2$O$_2$ treatment, 10% MTT (Sigma Aldrich, USA) was added to the culture medium of DMEM/F12 + 2% FBS, 0.5 mM L-Glutamine (Invitrogen, USA), and 1% streptomyacin/penicillin (Sigma Aldrich, USA), supplied with 5% CO$_2$. To induce hypoxia-like apoptosis, 100 μM H$_2$O$_2$ was added into cardiomyocyte culture for 6 h, 12 h, 24 h or 27 h.

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-$\Delta$ΔCt) 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$_2$O$_2$ 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 horse-radish 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.
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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\textsubscript{2}O\textsubscript{2} induced cytotoxicity and upregulated miR-100 in cardiomyocytes in time-dependent manner

We first examined the effect of H\textsubscript{2}O\textsubscript{2}-induced cytotoxicity in cardiomyocytes. We cultured mouse primary cardiomyocytes in vitro and treated them with various durations of 100 μM H\textsubscript{2}O\textsubscript{2} to induce hypoxia-like cytotoxicity. At 6, 12, 24 and 72 hours after H\textsubscript{2}O\textsubscript{2} treatment, the cytotoxicity was measured by a cell viability assay (*P<0.05). We also examined the gene expression levels of miR-100 in response to H\textsubscript{2}O\textsubscript{2} treatment. The results demonstrated miR-100 was significantly upregulated by 6, 12, 24 or 72 hours treatment of 100 μM H\textsubscript{2}O\textsubscript{2}, also in a time-dependent manner (*P<0.05).

Inhibition of miR-100 reduced H\textsubscript{2}O\textsubscript{2}-induced cardiomyocyte apoptosis

Since we found miR-100 was upregulated by H\textsubscript{2}O\textsubscript{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\textsubscript{2}O\textsubscript{2} for additional 24 hours, followed by a TUNEL immunostaining assay to examine the effect of miR-100 down-regulation on H\textsubscript{2}O\textsubscript{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\textsubscript{2}O\textsubscript{2} induced significant apoptosis in cardiomyocytes as more TUNEL-positive cells were...
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observed (Figure 2A, H₂O₂/miR-NC vs. Control), down-regulation of miR-100 significantly reduced the TUNEL-positive cardiomyocytes (Figure 2A, H₂O₂/miR-100-Inhibitor vs. H₂O₂/miR-NC). Further measurement on the percentage of healthy (TUNEL-negative) cardiomyocytes verified the protective effect of down-regulating miR-100 on H₂O₂-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 downstream signaling pathways involved in miR-100 regulation on H₂O₂-induced cytotoxicity in cardiomyocytes. Western blotting analysis demonstrated that IGF1R was upregulated while miR-100 was down-regulated during H₂O₂-induced apoptosis (Figure 3A). Thus, we hypothesized that IGF1R may directly mediate the protection of miR-100 downregulation on H₂O₂-induced cytotoxicity in cardiomyocytes. To examine that, we conducted the experiment of double-transfections on cardiomyocytes before H₂O₂ 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 H₂O₂ for another 24 hours. Third, 12 hours before H₂O₂ 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 downregulation on H₂O₂-induced cytotoxicity in cardiomyocytes.

Discussion

In the present study, we demonstrated that cultured mammalian cardiomyocytes were significantly injured by 100 μM H₂O₂ 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].
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Also in the present study, we demonstrated that miR-100 was upregulated by H$_2$O$_2$-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$_2$O$_2$-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$_2$O$_2$-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$_2$O$_2$-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$_2$O$_2$-induced cardiac cytotoxicity and apoptosis. Our work may provide new clinical strategy to treat...
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patients with conditions of acute myocardial infarction.

Disclosure of conflict of interest

None.

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