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Endogenous microRNAs induced by heat-shock reduce myocardial infarction following ischemia–reperfusion in mice

Chang Yin, Xiaoyin Wang, Rakesh C. Kukreja*

Division of Cardiology, Department of Internal Medicine, Virginia Commonwealth University Medical Center, 1101 E. Marshall St. Sanger Hall, Box 980281, Richmond, VA 23298-0281, USA

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Abstract **We investigated the role of microRNAs (miRNA) in protection against ischemia/reperfusion (I/R) injury in heart. Mice subjected to cytoprotective heat-shock (HS) showed a significant increase of miRNA-1, miRNA-21 and miRNA-24 in the heart. miRNAs isolated from HS mice and injected into non-HS mice significantly reduced infarct size after I/R injury, which was associated with the inhibition of pro-apoptotic genes and increase in anti-apoptotic genes. Chemically synthesized miRNA-21 also reduced infarct size, whereas a miRNA-21 inhibitor abolished this effect. Overall, these studies for the first time provide evidence for the potential role of endogenously synthesized miRNA's in cardioprotection following I/R injury.**

Keywords: Microrna; Heat-shock; Myocardial infarction; Apoptosis

1. Introduction

MicroRNAs (miRNAs) are family of small regulatory molecules that function by modulating protein production. There are approximately 500 known mammalian miRNA genes, and each miRNA may regulate hundreds of different protein-coding genes. miRNA biogenesis starts in the nucleus where miRNA is transcribed by RNA polymerase II to generate long primary transcripts (pri-miRNA). The pri-miRNA is trimmed by RNase III type enzyme drosha to release the hairpin intermediates (pre-miRNA). The pre-miRNA is then exported to the cytoplasm by expotin-5 where they are subjected to the second processing by Dicer, the cytoplasmic RNase III type enzyme. The pre-miRNA is cleaved into the short-lived miRNA duplex, whose one strand is degraded by an unknown nuclease while the other strand remains as a mature miRNA [1–5]. Binding with miRNAs in the cytoplasm is responsible for negative regulation of the target either through degradation of the bound mRNA or by inhibition of its translation [6]. Therefore, up-regulation of miRNAs leads to decreased gene expression. However, they can also lead to up-regulation of proteins by negatively modulating the expression of inhibitory genes.

Recent studies suggest that miRNA participate in many cellular processes, such as apoptosis [7–9], fat metabolism [10], cell differentiation [11–13], tumorigenesis [14] and cardiogenesis [15–19]. miRNAs are also critically involved in the pathological process of adult hearts, including cardiac hypertrophy [20–23], heart failure [24], angiogenesis [25] and arrhythmogenesis [26]. However, the potential role of endogenously synthesized miRNAs in attenuation of myocardial ischemia/reperfusion injury by well-established endogenous therapeutic has never been studied.

It has been shown that exposing hearts to stresses such as sub-lethal ischemia or mild heat-shock improves myocardial survival after subsequent prolonged ischemia/reperfusion injury [27–29]. Molecular chaperones that are rapidly synthesized and deployed to prevent protein misfolding and to assist in their refolding to the native state [30]. A set of genes and signaling pathways involved in heat-shock-induced protection have been proposed [31,32]. However, the regulation of cardioprotection following heat-shock at the pretranslational level has never been investigated. In the present study, we tested the hypothesis that miRNA may play an important role in protection against ischemia/reperfusion injury in the heart. We induced endogenous miRNA through heat-shock and injected them into non-heat-shocked mice. This experimental design took advantage of testing the role of miRNA using the animal's own endogenously induced miRNA in vivo. Our results show that miRNA reproduced heat-shock like protection against ischemia/reperfusion injury in the non-heat-shocked mice, apparently through mechanisms involving repression of apoptotic genes and upregulating anti-apoptotic genes.

2. Materials and methods

2.1. Animals

Adult outbred ICR mice from Harlan (Indianapolis, Indiana) were used and the guidelines on humane use and care of laboratory animals for biomedical research published by NIH (No. 85-23, revised 1996) were strictly complied for all animal experiments.

2.2. miRNA induction

The mouse was anaesthetized with sodium pentobarbital (50 mg/kg body weight, i.p.). Approximately 10 min after the injection, the animal was placed on an electric heating pad which was folded to cover up the whole body except head. A small diameter rectal thermal probe (YSI-402) was inserted into the animal's colon (about 1 cm) to record the core body temperature. The animals were then subjected to heat-shock by raining the temperature to 42 °C for 15 min. Animals in the sham control groups received identical treatment except their body temperature was not raised. After their recovery at room temperature for 2 h, the hearts and livers were removed for isolation of miRNA. Since whole body heat-shock also affects liver in terms of the synthesis of heat-shock proteins and inducing ischemic tolerance [48], we used both liver as well as heart for extraction of miRNAs in order to have sufficient amount of miRNA for in vivo treatments.

*Corresponding author. Fax: +1 804 828 870.
E-mail address: rakesh@vcu.edu (R.C. Kukreja).

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2.3. miRNA verification

miRNA was isolated from the hearts of both heat-shocked and non-heat-shocked mice using a miRNA Isolation Kit from Ambion (Austin, TX, USA). The isolation method combines the chemical and solid phase extraction techniques to obtain optimal miRNA. The isolated miRNA was treated with DNase to eliminate DNA contamination (DNA-free™, Ambion) and confirmed by RT-PCR using specific primers to miRNA 1, 21 and 24 (Ambion). RT-PCR was performed using Ambion's miRNA Detection Kit. The RT-PCR amplified miRNA was visualized on 3.5% high resolution agarose gel and measured by densitometer. miRNA signals from both treated and non-treated mice were normalized by GAPDH from the same samples to eliminate loading error.

2.4. miRNA treatment

Prior to injection, the isolated miRNAs were incubated in polyamine solution at 22 °C for 30 min to form miRNA–amine complexes [49]. The complex containing 40 μg miRNA was then injected intraperitoneally into the non-heat-shocked mice. To verify the specific role of miRNA's, a group of mice were treated with chemically synthesized miRNA-21 to reproduce the results obtained by utilizing heat-shock-induced miRNAs. Another sub-set of mice were treated with miRNA-21 with and without antisense miRNA-21 to see if the infarct limiting effect of miRNA-21 is abolished. The modified antisense oligonucleotide (2′OMe-miR-21), also called miRNA inhibitor [50], had the following sequence and structure: 5′-mUmCmAmAmCmAmUmCmAmGmUmCmU–mGmAmUmAmAmGmCmA-3′.

2.5. Langendorff isolated perfused heart preparation

Twenty-four hours after miRNA injection, the animals were re-anesthetized with sodium pentobartital (100 mg/kg with 33 IU heparin, i.p.). The heart was then removed quickly from the thorax and dropped into a small dish containing ice-cold Krebs–Henseleit solution with heparin. Under an illuminated magnifier, the aortic opening of mouse heart was immediately cannulated and tied on a 20 gauge stainless steel blunt needle which was connected to a perfusion system in Langendorff mode. Hearts were retrogradely perfused with a modified Krebs–Henseleit solution (contained NaCl 118, $NaHCO_3$ 24, $CaCl_2$ 2.5, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, Glucose 11, EDTA 0.5, in mM; gassed with 95% O_2 + 5% CO_2; pH 7.39–7.42) at a constant pressure of 55 mmHg. The perfusion solution was warmed through a water-jacketed glass cylinder/heat exchanger system and the temperature was monitored continuously by a thermocouple thermometer (COLE-PALMER, Model 8112-10) with a Type K micro-probe and maintained at 37 ± 0.2 °C throughout the experiment. Hearts were subjected to 20 min of global ischemia followed by reperfusion fro 30 min. At the end of reperfusion, the heart was immediately removed from the Langendorff apparatus, weighed and frozen at −20 °C. The frozen heart was cut into six to seven transverse slices, stained by 10% tetrazolium chloride for 30 min at room temperature (~22 °C) and subsequently fixed with 10% formalin for 2–4 h. The infarct area and risk zone was measured using computer morphometry (Bioquant 98). The risk area was calculated as total ventricular area minus the area of the cavities. The infarct size was presented as percentage of the risk area.

2.6. Gene microarray analysis

Effect of miRNA treatment on apoptotic genes was assessed using cDNA array containing 112 key genes involved in apoptosis. Total RNA from both miRNA injected and control mouse hearts was isolated and incubated with DNase to eliminate DNA contamination. Thereafter, mRNA was reverse transcribed, labeled with biotin-UTP, hybridized with the array as described by Superarray Bioscience Corporation (Fredrick, MD, USA). The hybridized signal was detected using Chemiluminescent Detection Kit from the same company.

2.7. Data analysis and statistics

All data were normalized by their corresponding control and presented as the group means ± S.E.M. The difference among experimental groups was compared by unpaired *t*-test or one-way ANOVA followed by Student–Newman–Keuls post-hoc test. $P < 0.05$ was considered as statistically significant.

3. Results

3.1. miRNA induction

Mice subjected to heat-shock showed induction in miRNA as compared to the non-heat-shocked control. The miRNA induction was verified using RT-PCR which detected significant increases in miRNA-1 (78%), miRNA-21 (103%) and miRNA-24 (61%) in the heart as shown in Fig. 1. However, only miRNA-1 was verified in the liver (not shown) although a number of other miRNAs may have been induced as well.

3.2. Infarct size

Mice treated with the mixture of miRNA isolated from heat-shocked mice demonstrated improved ischemic tolerance. Infarct size was reduced significantly e from 40 ± 2.7 (percentage of total risk area, mean ± S.E.M.) in the non-heat-shocked controls to 18.5 ± 3.8 in mice treated with the miRNA (Fig. 2). Moreover, chemically synthesized exogenous miRNA-21 also reduced infarct size by 64% ($P < 0.05$ versus control). The miRNA-21 induced protection was totally

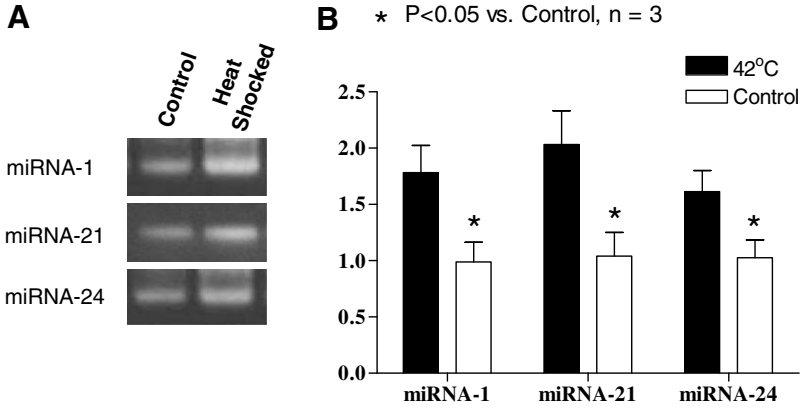

Fig. 1. Induction of miRNA following heat-shock in mice. (A) RT-PCR products of miRNA were visualized in 3% high resolution agarose gel. (B) Statistical analysis of miRNA following heat-shock. Data are normalized first by GAPDH and then by control. miRNA was induced by heat-shock and extracted 2-h later. The extracted miRNA was reverse transcribed into cDNA and amplified using PCR-primers from Ambion. The PCR condition used was: 30 cycles of 94 °C/30 s, 55 °C/30 s and 72 °C/30 s.

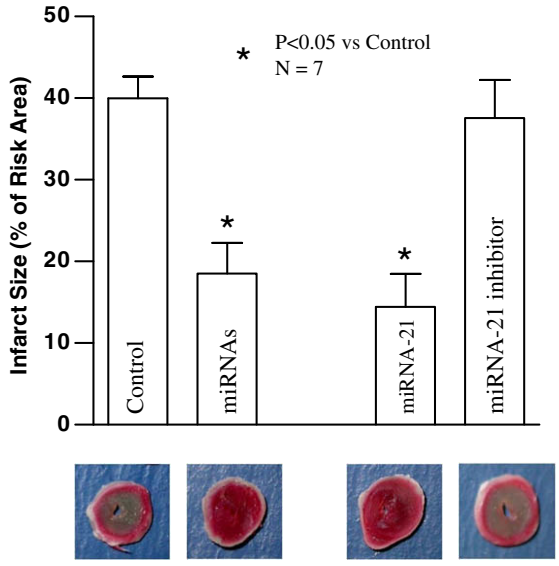

Fig. 2. Effect of miRNA-21 and its inhibitor on cardiac infarct size following ischemia/reperfusion. Top: bar diagram showing a significant reduction of infarct size compared to the non-treated controls. Chemically synthesized miRNA-21 also reduced infarct size significantly. The miRNA induced infarct size reduction was completely abolished by miRNA-21 inhibitor. Bottom: representative images of mouse heart slices stained with 10% of triphenyl tetrazolium chloride (TTC). Heart samples were selected from the following experimental groups: Control, miRNAs, miRNA-21 and miRNA-21 inhibitor. Note that infarct area is significantly larger (as shown by the increased pale areas) in the control and miRNA-21 inhibitor treated mice as compared to the miRNAs and miRNA-21 groups. The hearts were subjected to 20 min of global ischemia and 30 min of reperfusion 24-h following miRNA injection. Infarct size was measured at the end of ischemia–reperfusion using tetrazolium staining.

abolished when mice were co-treated with the miRNA-21 inhibitor.

3.3. miRNA and apoptosis

miRNA treatment caused profound changes in several apoptotic related genes as determined by gene microarray analysis. As shown in Fig. 3A, the caspase family members 1, 2, 8 and 14 were suppressed in the hearts treated with miRNA from heat-shock mice as compared to the controls. Except for BNIP-3, most of the pro-apoptotic genes including Bid (BH3 interacting domain death agonist), Bcl-10 (B-cell leukemia/lymphoma 10), Cidea (cell death-inducing DNA fragmentation factor, alpha subunit-like effector A), Ltbr (lymphotoxin B receptor), Trp53 (transformation related protein 53), Fas (TNF receptor superfamily member) and Fasl (Fas ligand, TNF superfamily, member 6), were also repressed (Fig. 3C). On the other hand, the anti-apoptotic genes, Bag-3 (Bcl-2-associated athanogene and Prdx2 (Peroxiredoxin 2) were increased (Fig. 3C).

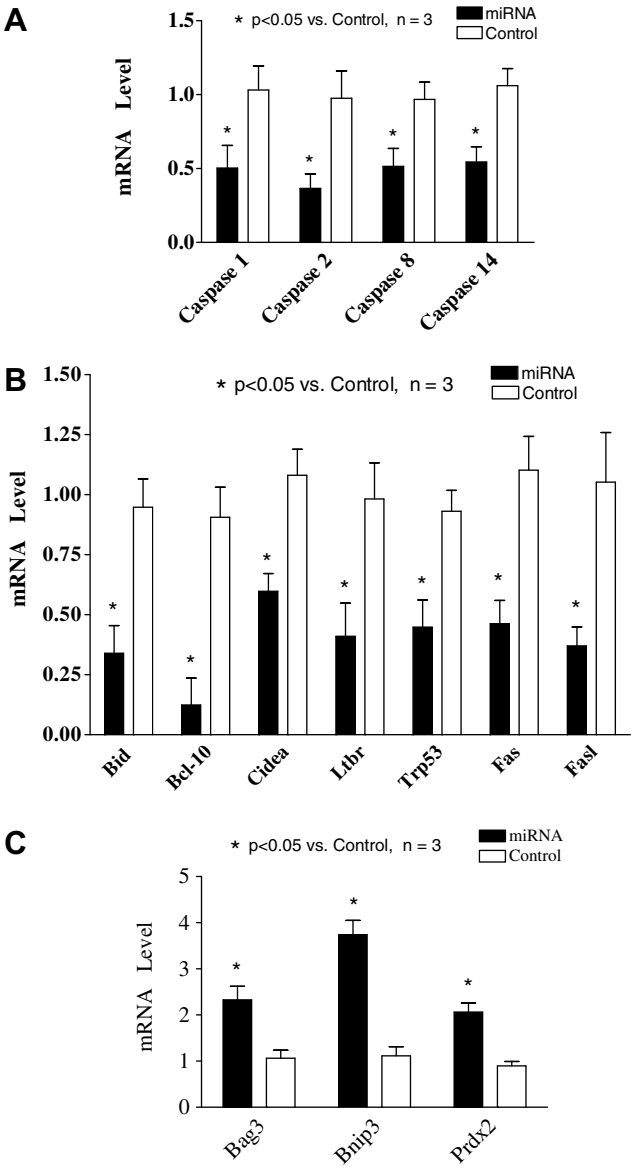

Fig. 3. Effect of miRNA treatment on apoptotic genes. (A) miRNA treated hearts demonstrated a depressed expression of caspase family members. (B) Except for Bnip-3miRNA treatment suppressed expression of several pro-apoptotic genes. (C) Increase in anti-apoptotic genes in the heart in miRNA treatment mice. Total RNA was extracted 4-h after miRNA injection and incubated with DNase to eliminate DNA contamination. The purified total RNA was then labeled and hybridized to apoptosis gene arrays following manufacturer's manual.

4. Discussion

Several studies have shown that heat-shock treatment protects the heart against ischemia/reperfusion injury [33]. The specific mechanisms underlying heat-shock protection include synthesis of heat-shock proteins [34], antioxidant defenses [35], and enhanced mitochondrial respiration [36]. In addition, it has been shown that heat-shock protects by opening of mitochondrial K_{ATP} channels [37] and causes resistance to opening of mitochondrial permeability transition pore [38], which may contribute to heat-shock protection against cellular injury through inhibition of apoptosis. In the present study, we have observed a significant induction of miRNA-1, miRNA-21 and miRNA-24 following whole body heat-shock in the heart. Moreover, mice treated with miRNAs isolated from the heat-shocked mice demonstrated significantly reduced infarct size in the heart following global ischemia and reperfusion.

Similarly, injection of chemically synthesized exogenous miRNA-21 reduced infarct size and the co-treatment with the 2′-O-methyl miRNA – which blocks miRNA-21 through antisense inhibition abolished the protective effect. Except for Bnip-3, miRNA injection caused downregulation of pro-apoptotic proteins including caspases 1, 2, 8 and 14, Bid, Bcl-10, Cidea, Ltbr, Trp53 and Fasl, while anti-apoptotic proteins including Bag-3, and Prdx2 were increased. These results suggest a potential role of miRNAs in reducing myocardial infarction through repression of apoptotic genes and up-regulation of anti-apoptotic proteins.

Although only three miRNA, namely miRNA-1, 21 and 24, were verified in the present study, heat-shock may well induce many other miRNAs. We did not perform experiments to demonstrate whether these intraperitoneally injected miRNAs ended up in the heart. Nevertheless, a recent study showed that the simple systemic delivery of a unconjugated locked-nucleic-acid-modified oligonucleotide (LNA-antimiR) effectively antagonized the liver-expressed miRNA-122 [39]. Acute administration by intravenous injections of LNA-antimiR in monkeys resulted in uptake of the LNA-antimiR in the cytoplasm of primate hepatocytes and formation of stable heteroduplexes between the antimiR and miRNA-122. This was accompanied by depletion of mature miRNA-122 and dose-dependent lowering of plasma cholesterol. Our data also supports these findings because the chemically synthesized miRNA-21 reduced infarct size in the heart which was blocked with miRNA-21 inhibitor. These data suggest the possibility that the physiological effect of the miRNA-21 and its antagonist were actually occurring in the heart following intraperitoneal injection.

Apoptosis is a major cause for cardiac infarction following ischemia–reperfusion [40,41]. miRNA-1 is preferentially expressed in cardiac muscle [42] and has been shown to regulate apoptosis. The inhibition of miRNA-21 has been shown to suppress cell growth by increasing apoptosis and decreasing cell proliferation [43]. In contrast, knockdown of miR-21 in cultured glioblastoma cells triggers activation of caspases and leads to increased apoptotic cell death [7]. miRNA-24 has recently been shown to be involved in the inhibition of skeletal muscle differentiation by TGF-β which provides clues for mechanisms underlying the physiological roles of the growth factor during myogenesis [44]. The attenuation of myocardial infarction with miRNA in this study may be related to reduced expression of apoptotic genes, Bid and Bcl-10 which may account for the observed protection since increased Bid and Bcl-10 can bind to Bcl-2 to promote apoptosis. On the other hand, Bag-3 may compete with Bid and Bcl-10 to bind to Bcl-2 to reduce apoptosis [45]. miRNA induction may also reduce infarct size through additional cellular processes other than apoptosis. For example, the increased levels of Prx2 observed in the current study may protect heart against oxidative stress since Prx2 is an extremely efficient scavenger of hydrogen peroxide [46].

In this study, Bnip-3 was increased in the heart following miRNA treatment. Although it is well recognized to be an apoptotic gene, some studies suggest that BNIP-3 is not sufficient for cell death but rather plays a critical role in hypoxia-induced autophagy [47]. Moreover, it has been suggested that rather than promoting death, BNIP-3 may actually allow survival either by preventing ATP depletion or by eliminating damaged mitochondria [47]. Such a function of BNIP-3 may be subverted under conditions associated with acidosis that arise following extended periods of hypoxia and anaerobic glycolysis. Bnip-3 is also shown to be expressed in healthy adult heart without evidence of cell death [48]. This finding is in line with the study by Tracy and Macleod, who found that Bnip-3 allowed cells to survive by preventing ATP depletion or by eliminating damaged mitochondria [47]. Overexpression of Bnip-3 in HL-1 cardiac myocytes subjected to simulated ischemia/reperfusion, caused up-regulation of autophagic activity which constituted a protective response against Bnip-3-mediated death signaling [49].

In conclusion, for the first time, we have provided evidence for the potential role of endogenously synthesized miRNAs in cardioprotection following ischemia/reperfusion injury. These miRNA have many advantages over other exogenous agents. For example, they are natural cellular products and therefore, non-toxic to cells. They can be induced in vivo under natural conditions, such as hyperthermia. Due to their short length, miRNAs can also easy to move around and cross sub-cellular structures. Therefore, identifying the role of endogenously synthesized miRNAs in protective pathophysiological stimuli including ischemic, heat-shock and by pharmacological preconditioning means may open up novel strategies to protect the heart in patients with coronary artery disease.

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