A Novel Role of MicroRNA in Late Preconditioning

Upregulation of Endothelial Nitric Oxide Synthase and Heat Shock Protein 70

Chang Yin,* Fadi N. Salloum,* Rakesh C. Kukreja

MicroRNAs (miRNAs) are noncoding RNAs of 18 to 24 nucleotides that are involved in posttranscriptional regulation of protein expression. Their role in ischemic preconditioning (IPC) is currently unknown. We hypothesized that miRNAs induced after IPC in the heart may create a preconditioned phenotype through upregulating proteins including endothelial nitric oxide synthase (eNOS)/inducible nitric oxide synthase (iNOS) and heat shock protein (HSP)70, which are implicated in the late-phase protection of IPC. miRNAs were extracted from hearts of ICR mice following IPC. The purified miRNAs were injected in vivo into the left ventricular wall of mice, and, 48 hours later, the hearts were subjected to regional ischemia/reperfusion injury by left anterior descending artery ligation for 30 minutes followed by reperfusion for 24 hour. IPC caused no changes in miRNA-23b and miRNA-483 whereas miRNA-1, miRNA-21and miRNA-24 were significantly increased. The IPC-miRNA treatment caused an increase in eNOS mRNA and protein, whereas iNOS was not changed. HSF-1 (heat shock transcription factor 1) and HSP70 were also increased with IPC-miRNA treatment versus control. Moreover, injection of IPC-miRNA protected the hearts against ischemia/reperfusion injury, as shown by a reduction of infarct size as compared with saline or non-IPC miRNA-treated control. We conclude that IPC-induced miRNAs trigger cardioprotection similar to the delayed phase of IPC, possibly through upregulating eNOS, HSP70, and the HSP70 transcription factor HSF-1.

Ischemic preconditioning (IPC) is a powerful cardioprotective phenomenon whereby repeated brief episodes of ischemia protect the heart against future myocardial infarction. Genetic reprogramming emerging during or following IPC, which simulates angina in the clinical setting can be characterized as protective in nature. Several mechanisms for IPC have been proposed, which broadly include the release of endogenous mediators including adenosine, activation of G-coupled receptors and protein kinase C, and synthesis of cytoprotective proteins including endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS), cyclooxygenase-2, and heat shock protein (HSP)70, which either individually or in concert lead to protection against ischemia/reperfusion (I/R) injury.

In recent years, microRNAs (miRNAs) have emerged as novel regulators of gene expression. The miRNAs are a family of small RNA with average length of 24 nucleotides that is too short to code for any proteins. For a long time, miRNAs were considered as byproducts of mRNA transcription, or even as “evolutionary transcriptional debris.” However, recent studies suggest that miRNAs participate in many cellular processes, such as apoptosis, fat metabolism, cell differentiation, tumorigenesis, and cardiogenesis. miRNAs are also critically involved in the pathological process of adult hearts, including cardiac hypertrophy, angiogenesis, arrhythmogenesis, and heart failure. We recently observed that whole body heat shock leads to synthesis of several miRNAs, which lead to protection against I/R injury. However, the potential role of endogenous miRNAs in IPC has never been investigated.

In the present study, we tested the hypothesis that miRNAs induced by IPC play an important role in protection against myocardial I/R injury. We induced miRNAs by short bursts of global ischemia and reperfusion in the isolated perfused hearts. The induced miRNAs (IPC-miRNAs) were then injected directly into the myocardium in vivo 48 hours before I/R injury. For the first time, our results show that miRNAs cause significant reduction in infarct size, which is associated with the upregulation of protective proteins including eNOS, HSF-1 (heat shock transcription factor 1), and HSP70 that are implicated in the delayed phase of IPC in the heart.

Materials and Methods

Details of the IPC/infarction protocol, miRNA isolation, their verification, and injection, as well as measurement of infarct size are provided in the online data supplement at http://circres.ahajournals.org.

Results and Discussion

In the Langendorff model, IPC stimulus (2 bursts of 30 seconds global ischemia followed by 90 seconds reperfusion; Figure 1A) reduced infarct size from 29.7±2.1% in control group to 9.1±1.8% in the IPC group (69.3% reduction, mean±SEM, P<0.05; Figure 1B). The IPC protocol caused no changes in miRNA-23b and miRNA-483 (Figure 1C) but caused a significant induction of miRNA-1 (162±13%), miRNA-21 (118±6%), and miRNA-24 (46±12%) as compared to control (Figure 1D). To determine the cause–effect relationship between IPC-induced endogenous miRNAs and cardioprotection, we injected the pool of extracted miRNAs from non-IPC and IPC hearts directly into the left ventricular wall in situ in a separate set of mice (miRNA-injected group). Forty-eight hours later, the mice were subjected to I/R injury in vivo by ligation of left

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coronary artery for 30 minutes followed by reperfusion for 24 hours. Our results show that miRNAs derived from IPC hearts produced a protective phenotype with significantly lower infarction (18.8±2.5%) as compared to saline-injected controls (37.5±2.2%) or miRNAs prepared from non-IPC hearts (39.3±2.3%). There was no difference in infarct size between saline-injected controls versus non-IPC miRNA-treated hearts. Also, there were no significant differences in risk areas between the groups (Figure 2B). To confirm the delivery of miRNA by intramyocardial injection, RT-PCR was performed and the results illustrate a significant uptake of miRNA-21 1 hour after injection in the risk zone (Figure 3A).

To gain further insight into the mechanisms underlying miRNA-induced protection, we probed several target protec-
tive proteins that are implicated in IPC, including eNOS, iNOS, HSP70, and the HSP70 transcription factor HSF-1. As shown in Figure 3A, induction of eNOS mRNA (61 ± 6.7%) was detected in the IPC-miRNA group 2 hours following treatment. However, no changes in iNOS mRNA were observed. Western blot analysis confirmed a significant upregulation in eNOS protein (62.0 ± 3.0%) and HSF-1 (42.7 ± 3.0%) 4 hours after IPC-miRNA treatment. HSP70 was also significantly increased (102.3 ± 8.9%) 48 hours after IPC-miRNA treatment. Again, similar to mRNA, iNOS protein was not significantly changed (Figure 3C and 3D).

Despite potential species differences in cardioprotection, it is widely known that the protective effects of IPC occur in an early phase that develops rapidly after the initial stimulus but dissipates within 2 to 3 hours and a late phase that becomes apparent 12 to 24 hours later and persists for ∼72 hours. The role of NO-derived from eNOS in the late phase of IPC has been suggested previously. Although the critical role of iNOS has also been well documented in the late phase of IPC and pharmacological preconditioning, we did not observe upregulation of this protein in the IPC-miRNA-treated hearts. This is possibly attributable to the strength of the IPC stimulus, which may not be sufficiently potent to trigger miRNA changes for synthesis of iNOS. A previous study has shown that 6 cycles of 4-minute coronary occlusion/4-minute reperfusion cause significant increase in myocardial iNOS. In this study, we used a less stringent IPC protocol, ie, 2 bursts of 30 seconds ischemia and 90 seconds reperfusion.

The induction of HSPs by stressful stimuli such as elevated temperature or ischemia is mediated by the HSP70 transcription factor HSF-1. It is known that the cytoplasmic HSF-1 monomer forms a trimer and moves to the nucleus, where it binds to its target sites (known as heat shock elements) in the regulatory regions of the HSP genes. Following its phosphorylation, HSF-1 induces expression of HSP70 that could protect hearts against ischemic injuries during the late phase of IPC. Our results show that IPC-miRNAs can induce HSF-1 and HSP70, which may play a role in ischemic tolerance observed in the heart. However, we do not know which particular miRNA is responsible for the increased synthesis of HSF-1/HSP70 or eNOS in the present.
miRNA-1 has been linked in posttranscriptional repression of HSP60 and HSP70 in H9c2 cells which is in contrast to the upregulation of HSP70 synthesis observed in the present study. Also, eNOS introns contain miRNAs which regulate eNOS expression. Although we observed a significant upregulation of miRNA-1, miRNA-21, and miRNA-24, it is possible that other miRNAs may have caused increased expression of the cytoprotective proteins as well. It is known that miRNAs generally function as inhibitory mechanisms of gene expression, and, therefore, it is possible that suppression of genes participating in injurious processes during I/R injury may underlie miRNA-induced protection in the heart. At the same time, it is possible that the suppression of injurious genes may lead to upregulation of protective proteins including eNOS and HSP70, as shown in the present study. Identification of such injurious genes is critical for validation of this hypothesis. Nevertheless, the protection observed against I/R injury in this study clearly suggests that concerted action of one or perhaps several miRNAs, induced following IPC, may have been responsible for the increased expression of eNOS and HSP70. Further studies are needed to identify the miRNA(s) from IPC that could increase the cytoprotective proteins without adverse side effects. The delivery of such miRNA(s) in the heart would have immense therapeutic potential in reducing myocardial infarction in patients with heart disease.

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**Disclosures**
None.

**References**

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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.

Langendorff Isolated Perfused Heart Preparation: The methodology of the Langendorff isolated perfused heart has been previously described in detail (1). In brief, the mice were anesthetized with an IP injection of sodium pentobarbital (100 mg/kg containing 33 international units of heparin). The hearts were removed and submerged in ice-cold Krebs-Henseleit (K-H) solution containing heparin. The aorta was secured to a 20 gauge stainless steel blunt needle, and the hearts were perfused at a constant temperature and pressure in a retrograde fashion via the aorta with K-H solution. Following a stabilization period of 30 minutes, I/R injury was produced by subjecting the hearts to 30 minutes of normothermic, no-flow global ischemia followed by reperfusion for 60 minutes. To obtain the developed pressure, a left atrial incision was made to expose the mitral annulas through which a water-filled latex balloon was passed into the left ventricle (LV). The balloon was attached via polyethylene tubing to a Gould pressure transducer that was connected to a PowerLab Acquisition System (ADInstruments 8SP, Australia). The balloon was inflated to adjust the LV end-diastolic pressure (LVEDP) to ~ 10 mmHg. Myocardial ischemic damage was measured using multiple, independent end points of tissue injury. These included infarct size, LV developed pressure (LVDP), LVEDP, rate-pressure product (RPP), heart rate, and coronary flow by timed collection of the perfusate. LVDP was calculated by subtracting LVEDP from the peak systolic pressure. RPP, an index of cardiac work, was calculated by
multiplying LVDP with heart rate. The hearts were not paced.

*Ischemic Preconditioning Protocol in Isolated Mouse Hearts:* The protocol for ischemic preconditioning (IPC) is similar to that described above. However, 4 min. prior to 30 min. of prolonged ischemia, the hearts are subjected to 2 cycles of 30 sec. ischemia followed by 90 sec. reperfusion.

*miRNA extraction, verification and treatment:* miRNAs were extracted from the hearts of different groups, using a miRNA isolation kit from Ambion (Austin, Texas). The isolation method combines the chemical and solid phase extraction techniques to obtain optimal miRNAs. The extracted miRNAs were first treated with DNase to eliminate DNA contamination (DNA-free™, Ambion). Then, the miRNAs were verified by RT-PCR using miRNA Detection Kit from Ambion. Prior to cardiac injection, miRNAs were incubated with polyamine at 22°C for 30 minutes to form miRNA-amine or miRNA inhibitor-amine complexes which facilitate miRNAs’ entry into cells (2,3,4). GAPDH RNA was used as control for miR-1, miR-21 and miR-24 and endogenous U1A small nuclear RNA (RNU1A) was used as control for miR-23b and miR-483 and for miR-21 to examine its uptake following injection.

*Injection of miRNA in the heart:* The animals were anesthetized with the injection of pentobarbital (70 mg/kg ip), intubated orotracheally and ventilated on a positive-pressure ventilator. The tidal volume was set at 0.2 ml, and the respiratory rate was adjusted to 133 cycles/min. All surgical procedures were carried out under sterile conditions. A left
thoracotomy was performed at the fourth intercostal space, and the heart was exposed by stripping the pericardium. Using 27G needles, three volumes of 10µl each containing 0.7 µg of miRNAs or PBS were injected into the myocardium of the left ventricle. After injection, the air was expelled from the chest. The animals were extubated and then received intramuscular doses of analgesia (buprenex; 0.02 mg/kg; sc) and antibiotic (Gentamicin; 0.7 mg/kg; IM, for 3 days). 48 h after injection, the myocardial infarction protocol was carried out.

Myocardial Infarction Protocol (in vivo): The methodology of in vivo myocardial infarction protocol has been previously described in detail (5). The animals were anesthetized with the injection of pentobarbital (70 mg/kg ip), intubated orotracheally and ventilated on a positive-pressure ventilator. The tidal volume was set at 0.2 ml, and the respiratory rate was adjusted to 133 cycles/min. All surgical procedures were carried out under sterile conditions. A left thoracotomy was performed at the fourth intercostal space, and the heart was exposed by stripping the pericardium. The left descending coronary artery was then identified and occluded by a 7.0 silk ligature that was placed around it for 30 min. followed by 24 h of reperfusion. At the onset of reperfusion, the air was expelled from the chest. The animals were extubated and then received intramuscular doses of analgesia (buprenex; 0.02 mg/kg; sc) and antibiotic (Gentamicin; 0.7 mg/kg; IM, for 3 days).

Infarct Size Assessment: After completion of the infarction protocol, the heart was quickly removed and mounted on a Langendorff apparatus. The coronary arteries were perfused with 0.9% NaCl containing 2.5 mM CaCl$_2$. After the blood was washed out, ~2 ml of 10% Evans
blue dye were injected as a bolus into the aorta until most of the heart turned blue. The heart was perfused with saline to wash out the excess Evans blue. Finally, the heart was removed, frozen, and cut into 6–8 transverse slices from apex to base of equal thickness (~1 mm). The slices were then incubated in a 10% TTC in isotonic phosphate buffer (pH 7.4) at room temperature for 30 min. The areas of infarcted tissue, the risk zone, and the whole left ventricle were determined by computer morphometry using a Bioquant imaging software.

Data Analysis and Statistics: All data were normalized by their corresponding control and presented as the group means ± standard error of mean. 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.

Reference List