MicroRNA-21 Mediates Isoflurane-induced Cardioprotection against Ischemia–Reperfusion Injury via Akt/Nitric Oxide Synthase/Mitochondrial Permeability Transition Pore Pathway

Shigang Qiao, M.D., Jessica M. Olson, Ph.D., Mark Paterson, B.S., Yasheng Yan, B.S., Ivan Zaja, B.S., Yanan Liu, Ph.D., Matthias L. Riess, M.D., Ph.D., Judy R. Kersten, M.D., Mingyu Liang, Ph.D., David C. Warltier, M.D., Ph.D., Zeljko J. Bosnjak, Ph.D., Zhi-Dong Ge, M.D., Ph.D.

ABSTRACT

Background: The role of microRNA-21 in isoflurane-induced cardioprotection is unknown. The authors addressed this issue by using microRNA-21 knockout mice and explored the underlying mechanisms.

Methods: C57BL/6 and microRNA-21 knockout mice were echocardiographically examined. Mouse hearts underwent 30 min of ischemia followed by 2 h of reperfusion in vivo or ex vivo in the presence or absence of 1.0 minimum alveolar concentration of isoflurane administered before ischemia. Cardiac Akt, endothelial nitric oxide synthase (eNOS), and neuronal nitric oxide synthase (nNOS) proteins were determined by Western blot analysis. Opening of the mitochondrial permeability transition pore (mPTP) in cardiomyocytes was induced by photoexcitation-generated oxidative stress and detected by rapid dissipation of tetramethylrhodamine ethyl ester fluorescence using a confocal microscope.

Results: Genetic disruption of miR-21 gene did not alter phenotype of the left ventricle, baseline cardiac function, area at risk, and the ratios of phosphorylated-Akt/Akt, phosphorylated-eNOS/eNOS, and phosphorylated-nNOS/nNOS. Isoflurane decreased infarct size from 54 ± 10% in control to 36 ± 10% (P < 0.05, n = 8 mice per group), improved cardiac function after reperfusion, and increased the ratios of phosphorylated-Akt/ACT, phosphorylated-eNOS/eNOS, and phosphorylated-nNOS/nNOS in C57BL/6 mice subjected to ischemia–reperfusion injury. These beneficial effects of isoflurane were lost in microRNA-21 knockout mice. There were no significant differences in time of the mPTP opening induced by photoexcitation-generated oxidative stress in cardiomyocytes isolated between C57BL/6 and microRNA-21 knockout mice. Isoflurane significantly delayed mPTP opening in cardiomyocytes from C57BL/6 but not from microRNA-21 knockout mice.

in the regulation of numerous physiologic and pathophysiologic processes. MicroRNA-21 (miR-21) is highly expressed in cardiomyocytes, cardiac fibroblasts, vascular endothelial cells, and vascular smooth muscle cells. The physiologic role of miR-21 in the cardiovascular system is incompletely known. There is evidence that miR-21 is involved in cardiac cell growth and death, regulation of cardiac fibroblast function, and vascular smooth muscle cell proliferation and apoptosis. However, the contribution of miR-21 to cardiovascular disease is increasingly identified, including myocardial infarction, atherosclerosis, myocardial fibrosis, cardiac hypertrophy, and heart failure. Recent studies indicate that miR-21 is closely associated with acute myocardial I/R injury. First, miR-21 in cardiomyocytes is up-regulated in response to hypoxia or ischemia. Second, down-regulation of miR-21 increases the vulnerability of myocardium to I/R injury, whereas increased expression of miR-21 enhances the tolerance of myocardium to I/R injury. Third, miR-21 inhibitor eliminates the cardioprotective effect of ischemic preconditioning. Recently, miR-21 is found to involve the ISO-induced protection of cardiomyocytes against hypoxia/reoxygenation injury. However, how miR-21 contributes to ISO-induced protection against myocardial I/R injury remains elusive. The aim of this study was to examine the role of miR-21 in ISO-induced protection against acute myocardial I/R injury using miR-21 gene knockout (KO) mice.

Materials and Methods

Animals
C57BL/6 wild-type (WT) mice and miR-21 KO mice (weight: 26.2 ± 1.9 g; age: 9 to 12 weeks) were purchased from The Jackson Laboratory (USA). The animals were kept on a 12-h light–dark cycle in a temperature-controlled room. The experimental procedures were approved by the Animal Care and Use Committee of the Medical College of Wisconsin (Milwaukee, Wisconsin) and conformed to the Guide for the Care and Use of Laboratory Animals (Institute for Laboratory Animal Research, National Academy of Sciences, 8th edition, 2011).

Hemodynamic Measurements
Mice were anesthetized by intraperitoneal injection of 80 mg/kg pentobarbital sodium and ventilated with room air supplemented with 100% O₂ at approximately 102 breaths/min. The right carotid artery was cannulated with a small polyethylene tubing catheter filled with 0.9% saline containing 10 U/ml heparin. The catheter was connected to an ADInstruments pressure transducer (MLT0380/D; ADInstruments, USA) and a Powerlab data acquisition system (ADInstruments). After a 30 min of stabilization, blood pressure was continuously recorded for 20 min. Body temperature was maintained between 36.8° and 37.5°C throughout the experiment by using a heating pad (Model TC-1000; CWE Inc., USA).

Transthoracic Echocardiography
Mice were sedated by the inhalation of ISO (1.50%) and oxygen. Noninvasive transthoracic echocardiography was performed with a VisualSonics Vevo 770 High-Resolution Imaging System (Canada) equipped with a 30-MHz transducer (Scanhead RMV 707; VisualSonics), as described previously. Left ventricular dimensions and ejection fraction were measured by two-dimensional–guided M-mode method. Pulsed Doppler waveforms recorded in the apical-4-chamber view were used for the measurements of the peak velocities of mitral E (early mitral inflow) and A (late mitral inflow) waves, E wave acceleration velocity, E wave acceleration time, E wave deceleration velocity, and E wave deceleration time, isovolumic contraction time, ejection time, and isovolumic relaxation time of left ventricle (LV). Myocardial performance index was calculated with the following formula: myocardial performance index = (isovolumic contraction time + isovolumic relaxation time)/ejection time.

Real-time Reverse Transcriptional Polymerase Chain Reaction
Pentobarbital-anesthetized mice were stabilized for 30 min and administered 1.0 minimum alveolar concentration (approximately 1.40% in the mouse) of ISO for 30 min via an ISO-specific vaporizer (Ohio Medical Instruments, USA). Control mice received no ISO. The heart was excised 30 min and 3 h after ISO treatment was discontinued, and the LV was homogenized at 4°C for real-time quantitative reverse transcriptional polymerase chain reaction (qRT-PCR) analysis of miR-21.

MiR-21 Extraction and qRT-PCR Analysis.
Total RNA from heart tissues was extracted using Qiagen reagent according to the protocol of the manufacturer (Qiagen, USA). Chloroform was added, and samples were centrifuged to facilitate phase separation. The aqueous phase was extracted and combined with ethanol in miRNeasy Mini spin columns (Qiagen). Total RNA was eluted in RNase-free water. The concentration of extracted total RNA was quantified by the Epoch spectrophotometer (Biotek, USA). Samples were considered pure if the A260/280 ratio was between 1.9 and 2.0. One microgram of total RNA from each sample was used to generate complementary DNA (cDNA) using miScript Reverse transcriptase mix, nucleic mix, and HiFlex Buffer (Qiagen). The cDNA product was measured in triplicate using miScript Primer Assays for miR-21 (Qiagen), qRT-PCR was conducted using the Bio-Rad iCycler real-time polymerase chain reaction (PCR) detection system (USA). Expression of miR-21 was normalized by expression of the housekeeping gene Rnu-6 (Qiagen). The relative gene expressions were calculated in accordance with the ΔΔCt method. Relative microRNA levels were expressed as percentages compared with non–ISO-exposed controls.

PCR Array of miR-21 Target mRNAs
C57BL/6 mice were given 1.0 minimum alveolar concentration of ISO for 30 min via an ISO-specific vaporizer.
(Ohio Medical Instruments). Control mice received no ISO. The heart was excised 30 min after ISO treatment was discontinued, and the LV was homogenized at 4°C for PCR array analysis of miR-21 target mRNAs using mouse miFinder RT² PCR array (SA Biosciences, USA) according to the manufacturer's instructions. These arrays investigate 84 experimentally validated and predicted targets of miR-21, including activity-dependent neuroprotector homeobox, centrosomal protein 68 kDa, de lin 1, eukaryotic translation initiation factor 4A2, krev interaction trapped protein 1, myristoylated alanine-rich C-kinase substrate, nuclear factor (NF) I/B, phosphatase and tensin homology deleted from chromosome 10 (PTEN), Ras homolog family member B (RHOB), ribosomal protein S7, reticulin-4, sprouty 2 (SPRY2), topoisomerase I binding, arginine/serine-rich, E3 ubiquitin protein ligase, tropomysin 1, ubiquitin-conjugating enzyme E2 D3, and Wolfram syndrome 1. Total RNA was extracted using the Qiagen RNeasy (Qiagen), and cDNA was prepared using the RT² First-Strand Kit (Qiagen). cDNA was diluted and combined with RT² SYBR Green/Fluorescein Master Mix and distributed evenly across the wells of a 96-well plate in technical triplicates. The BioRad iCycler Real-Time PCR Detection System was used in qRT-PCR analysis of miR-21 target mRNAs. Samples were exposed to an initial 95°C hot-start activation step, followed by 40 cycles of 94°C denaturation and 60°C annealing/extension phases. Data collected from these experiments defined Ct values of the mRNAs present in each sample. Expression levels of the housekeeping genes β-actin and glyceraldehyde 3-phosphate dehydrogenase were used as control to normalize samples. Samples without reverse transcriptase were run to confirm that no genomic DNA was present in the sample. mRNAs present in each sample. Expression levels of the housekeeping genes β-actin and glyceraldehyde 3-phosphate dehydrogenase were used as control to normalize samples.

Myocardial I/R Injury Ex Vivo

Myocardial I/R injury was produced by occluding the left coronary artery, as previously described.12,24 MiR-21 KO and WT-I/R injury was produced by occluding the left coronary artery at the site of previous occlusion. ISO = isoflurane; KO = knockout; miR-21 = microRNA-21; WT = wild type.

Fig. 1. Schematic diagram depicting the experimental protocols of myocardial ischemia–reperfusion (I/R) injury in vivo. ISO = isoflurane; KO = knockout; miR-21 = microRNA-21; WT = wild type.
Measurements of Mitochondrial Nicotinamide Adenine Dinucleotide Levels in Langendorff-perfused Mouse Hearts

Nicotinamide adenine dinucleotide (NADH) emits fluorescence when mitochondria are illuminated at the appropriate wavelength (for example, 350 nm, 460 nm, and so on). To measure NADH fluorescence in mouse hearts, Langendorff-perfused mouse hearts were placed within a light-proof Faraday cage to block incident room light, as described. A fiber-optic cable placed against the LV of Langendorff-prepared mouse hearts to excite and record transmyocardial fluorescence at a wavelength of 456 nm during ischemia and reperfusion. The two proximal ends of the fiber-optic cable were connected to a modified spectrophotofluorometer (Photon Technology International, Canada). Fluorescence \( F \) was excited with light at the appropriate wavelength \( \lambda \) from a xenon arc lamp at 75 W filtered through a monochromator (Delta RAM; Photon Technology International). NADH signal was recorded continuously using a Powerlab data acquisition system (ADInstruments).

Immunoblotting

Pentobarbital-anesthetized mice were subjected to myocardial I/R injury in vivo in the presence or absence of ISO, as illustrated in figure 1. The myocardium from the area at risk of mouse hearts was harvested and homogenized in a buffer containing 20.0 mM 3-(N-morpholino)propanesulfonic acid, 2.0 mM EGTA, 5.0 mM EDTA, protease inhibitor cocktail (1:100; Calbiochem, USA), phosphatase inhibitor cocktail (1:100; Calbiochem), and 0.5% detergent (Nonidet P-40 detergent pH 7.4; Sigma-Aldrich, USA). Immunoblots were performed using standard techniques, as described. In brief, tissue homogenates that contained 50 μg (for Akt or endothelial nitric oxide synthase [eNOS]) or 100 μg (for neuronal nitric oxide synthase [nNOS]) of protein were applied to 7.5% sodium dodecyl sulfate–polyacrylamide gel and subjected to immunoblot analysis by incubation with a mouse anti-Akt antibody (Cell Signaling, USA), a mouse anti-phosphorylated Akt (p-Akt) antibody (serine 473; Cell Signalling), an anti-eNOS antibody (Santa Cruz Biotechnologies, USA), an anti-phosphorylated eNOS (p-eNOS) antibody (serine 1177; Cell Signaling), an anti-nNOS antibody (Invitrogen), or an anti-phosphorylated nNOS (p-nNOS) antibody (serine 1412; Affinity Bioreagents, USA) at 4°C. The membrane was washed and then incubated with the appropriate anti-mouse secondary antibody. Immunoreactive bands were visualized by enhanced chemiluminescence followed by densitometric analysis using image acquisition and analysis software (Image J; National Institutes of Health, USA).

Detection of Opening of the Mitochondrial Permeability Transition Pore in Cardiomyocytes

Isolation of Cardiomyocytes. Cardiomyocytes were isolated from adult mice using the methods established by the Alliance for Cellular Signaling (http://www.signaling-gateway.org; protocol no. PP00000015). In brief, mouse hearts were cannulated via the aorta onto a blunted 20-gauge needle and perfused for 10 min at 37°C with perfusion buffer (113 mM NaCl, 4.7 mM KCl, 0.6 mM KH₂PO₄, 0.6 mM Na₂HPO₄, 1.2 mM MgSO₄·7H₂O, 0.032 mM phenol red, 12 mM NaHCO₃, 10.0 mM KHCO₃, 10.0 mM HEPES, pH 7.4, 30 mM taurine, 10 mM 2,3-butanedione monoxime, and 5.5 mM glucose) containing 0.25 mg/ml Liberase Blendzyme I, 0.14 mg/ml trypsin, and 12.5 μM CaCl₂. After perfusion, the LVs were dissected free from the atria and repeatedly passed through a plastic transfer pipette to disaggregate the cells into a single-cell suspension. Subsequently, myocytes were enriched by sedimentation in perfusion buffer containing 5% bovine calf serum while slowly exposing the cells to increasing concentrations of CaCl₂ to achieve a final concentration of 1.2 mM. The final cell
pellet containing calcium-tolerant myocytes was resuspended in the culture media containing Hanks salts, 2 mM l-glutamine, 5% bovine calf serum, 10 mM 2,3-butanedi-one monoxygenase, and 100 U/ml penicillin. After isolation, the myocytes were stored in Tyrode solution (132 mM NaCl, 10 mM HEPES, 5 mM glucose, 5 mM KCl, 1 mM CaCl₂, and 1.2 mM MgCl₂; adjusted to pH 7.4). Experiments were conducted at room temperature within 5 h after isolation using Tyrode solution.

**Experimental Protocol.** Cardiomyocytes isolated from mouse hearts were assigned to the following four groups (12 cells per group): WT-oxidative stress (OS), miR-21 KO-OS, WT-OS+ISO, and miR-21 KO-OS+ISO. The myocytes in the WT-OS and WT-OS+ISO groups were from C57BL/6 mice, and those in the miR-21 KO-OS and miR-21 KO-OS+ISO groups from miR-21 KO mice. After 20 min of stabilization at room temperature, the myocytes were incubated with Tyrode solution containing 0.5 mM ISO for 10 min followed by washout for 5 min or without ISO.

**Detection of Mitochondrial Permeability Transition Pore Opening.** A laser-scanning confocal microscope (Nikon Eclipse TE2000-U; Nikon Instruments Inc., USA) was used to measure opening of the mitochondrial permeability transition pore (mPTP) in cardiomyocytes. Opening of the mPTP was induced by photoexcitation-generated OS and detected by rapid dissipation of tetramethylrhodamine ethyl ester fluorescence (TMRE), as described. All cells were loaded with 50 nM TMRE at room temperature for 20 min. After TMRE loading, the cells were washed out for 5 min, and a recording region of 30 x 30 μm was exposed to narrowly focused laser scanning. Only cells with equal initial TMRE fluorescence intensity were included in the study, and the settings of the confocal microscope were consistent to ensure equal delivery of OS. Arbitrary mPTP opening time was determined as the time of loss of average TMRE fluorescence intensity from the recorded region (excluding nucleus) by half between initial and residual fluorescence intensity. It corresponded to complete depolarization of 50% of mitochondria in the recorded region.

**OS in Cardiomyocytes**
Cardiomyocytes were isolated from 1- to 2-day-old Sprague–Dawley rat pups using serial digestion with collagenase and pancreatin (Sigma-Aldrich), as described. Cardiomyocytes were grouped (n = 3 dishes per group) and received treatment as the following: (1) control: the cells were cultured for 7 days in full supplement plating medium; (2) green fluorescence protein (GFP): the cells were cultured for 4 days and transduced with 100 multiplicity of infection (MOI) of GFP-labeled adenovirus for 72 h; (3) pre-miR-21: the cells were cultured for 4 days and transduced with 100 MOI of pre-miR-21 adenovirus (Applied Biological Materials, Inc., Canada) for 72 h; (4) anti-miR-21: the cells were transduced with 100 multiplicity of infection (MOI) of GFP-labeled anti-miR-21 (Applied Biological Materials, Inc.) for 72 h without H₂O₂ treatment; (5) H₂O₂: the cells were cultured for 7 days in full supplement medium and exposed to 50.0 μM H₂O₂ (Calbiochem) for 4 h; (6) GFP+H₂O₂: the cells were cultured for 4 days, transduced with 100 MOI of GFP-labeled adenovirus for 72 h, and exposed to 50.0 μM H₂O₂ for 4 h; (7) pre-miR-21+H₂O₂: the cells were cultured for 4 days, transduced with 100 MOI of pre-miR-21 adenovirus for 72 h, and exposed to 50.0 μM H₂O₂ for 4 h; (8) anti-miR-21+H₂O₂: the cells were transduced with GFP-labeled anti-miR-21 for 72 h and exposed to H₂O₂ for 4 h. In all transduction experiments, transduction was visually confirmed through fluorescence microscopy, as well as through qRT-PCR analysis. Cell nuclei were stained with Hoechst 33342 (Life Technologies, USA) and confirmed an approximately 70 to 80% transduction efficiency in cardiomyocytes.

**Propidium Iodide Staining.** Nuclei were stained with Hoechst 33342 (Life Technologies) at a concentration of 1:800 in diluent cell culture media. Propidium iodide (PI) solution (Life Technologies) at 1.0 mg/ml was diluted in media at a concentration of 1:500. Hoechst-positive nuclei were counted as a control using fluorescence microscopy, and PI-positive cells were taken as a percentage of the control.

**Statistical Analysis**
Mice were randomly assigned to ISO-treated or control groups. Western blot and qRT-PCR analyses were conducted blindly, with samples divided into randomly numbered groups. All data are expressed as mean ± SD. Two-way repeated-measures ANOVA test was used to evaluate the differences in body weight, mean arterial blood pressure, heart weight, ratio of heart/body weight, and echocardiographic data. Statistical analysis of heart rate, ±dP/dt, and NADH over time between groups was performed with repeated-measures ANOVA followed by Bonferroni multiple comparison. One-way ANOVA followed by Bonferroni post hoc test was used to analyze the area at risk, the infarct size, the ratios of p-Akt/Akt, p-eNOS/eNOS, and p-nNOS/nNOS, and the mPTP opening time. All statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, Inc., USA). A value of P less than 0.05 (two tailed) was considered statistically significant.

**Results**

**Characteristics of C57BL/6 and miR-21 KO Mice**
Baseline characteristics of C57BL/6 and miR-21 KO mice are listed in table 1. There were no significant differences observed in body weight, mean arterial blood pressure, heart weight, and the dimension and function of the LV between miR-21 KO and C57BL/6 mice.

**Regulation of miR-21 and miR-21 Target mRNAs after ISO Treatment**
Figure 3 shows the expression of myocardial miR-21 mRNA and miR-21 target mRNAs in C57BL/6 mice after ISO treatment. The expression of miR-21 gene was significantly
Table 1. Basic Characteristics of C57BL/6 and miR-21 Knockout Mice

<table>
<thead>
<tr>
<th>Parameters</th>
<th>C57BL/6</th>
<th>miR-21 Knockout</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>General characteristics</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight, g</td>
<td>25.7 ± 1.8</td>
<td>6.7 ± 1.9</td>
</tr>
<tr>
<td>Mean arterial blood pressure, mmHg</td>
<td>97 ± 26</td>
<td>93 ± 23</td>
</tr>
<tr>
<td>Heart weight, mg</td>
<td>135 ± 13</td>
<td>129 ± 12</td>
</tr>
<tr>
<td>Heart/body weight, mg/g</td>
<td>5.1 ± 0.7</td>
<td>5.0 ± 0.1</td>
</tr>
<tr>
<td><strong>Echocardiographic parameters</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart rate, beats per minute</td>
<td>452 ± 61</td>
<td>438 ± 36</td>
</tr>
<tr>
<td>Anterior wall at end-diastole, mm</td>
<td>0.81 ± 0.11</td>
<td>0.84 ± 0.11</td>
</tr>
<tr>
<td>Anterior wall at end-systole, mm</td>
<td>1.28 ± 0.22</td>
<td>1.32 ± 0.13</td>
</tr>
<tr>
<td>Posterior wall at end-diastole, mm</td>
<td>0.87 ± 0.09</td>
<td>0.82 ± 0.13</td>
</tr>
<tr>
<td>Posterior wall at end-systole, mm</td>
<td>1.31 ± 0.28</td>
<td>1.12 ± 0.20</td>
</tr>
<tr>
<td>LV end-diastolic volume, μl</td>
<td>56 ± 12</td>
<td>63 ± 10</td>
</tr>
<tr>
<td>LV end-systolic volume, μl</td>
<td>18 ± 9</td>
<td>22 ± 5</td>
</tr>
<tr>
<td>Ejection fraction, %</td>
<td>70 ± 11</td>
<td>65 ± 6</td>
</tr>
<tr>
<td>Peak E wave velocity, cm/s</td>
<td>77 ± 15</td>
<td>67 ± 6</td>
</tr>
<tr>
<td>Peak A wave velocity, cm/s</td>
<td>45 ± 13</td>
<td>48 ± 4</td>
</tr>
<tr>
<td>Peak E/A ratio</td>
<td>1.79 ± 0.38</td>
<td>1.40 ± 0.18</td>
</tr>
<tr>
<td>Isovolumic contraction time of LV, ms</td>
<td>15.3 ± 4.9</td>
<td>17.5 ± 3.4</td>
</tr>
<tr>
<td>Isovolumic relaxation time of LV, ms</td>
<td>16.7 ± 2.1</td>
<td>15.6 ± 1.8</td>
</tr>
<tr>
<td>Myocardial performance index</td>
<td>0.72 ± 0.13</td>
<td>0.65 ± 0.11</td>
</tr>
<tr>
<td>Mitral E acceleration, cm/ms</td>
<td>8,295 ± 2,291</td>
<td>8,602 ± 1,002</td>
</tr>
<tr>
<td>Mitral E wave acceleration time, ms</td>
<td>8.9 ± 2.2</td>
<td>7.9 ± 1.0</td>
</tr>
<tr>
<td>Mitral E deceleration, cm/ms</td>
<td>4,425 ± 1,603</td>
<td>4,440 ± 954</td>
</tr>
<tr>
<td>Mitral E wave deceleration time, ms</td>
<td>16.5 ± 6.6</td>
<td>17.5 ± 2.8</td>
</tr>
</tbody>
</table>

There were no significant differences in all parameters between miR-21 knockout and C57BL/6 mice (n = 10 to 16 mice per group).

LV = left ventricle; miR-21 = microRNA-21.

Fig. 3. Regulation of cardiac microRNA-21 (miR-21) and miR-21 target mRNAs by isoflurane (ISO) in C57BL/6 mice. (A) Up-regulation of miR-21 mRNA by ISO. Pentobarbital-anesthetized mice received 1.0 minimum alveolar concentration of ISO for 30 min or oxygen–air mixture as control (no ISO). The expression of myocardial miR-21 gene was measured by real-time quantitative reverse transcriptional polymerase chain reaction 30 min and 3 h after the treatment of ISO or oxygen–air mixture. *P < 0.05 versus no ISO (n = 6 to 8 mice per group). (B) Regulation of miR-21 target mRNAs by ISO. MiR-21 target mRNAs were investigated using mouse miFinder RT2 polymerase chain reaction array 30 min after ISO or oxygen–air mixture as control (no ISO). ADNP = activity-dependent neuroprotective homeobox; CEP68 = centrosomal protein 68 kDa; DERL1 = derlin 1; EIF4A2 = eukaryotic translation initiation factor 4A2; KRIT1 = krev interaction trapped protein 1; MARCKS = myristoylated alanine-rich C-kinase substrate; NFIB = nuclear factor I/B; PTEN = phosphatase and tensin homology deleted from chromosome 10; RHOB = Ras homolog family member B, RPS7 = ribosomal protein S7; RTN4 = reticulin-4; SPRY2 = sprouty 2; TOPORS = topoisomerase I binding, arginine/serine-rich, E3 ubiquitin protein ligase; TPM1 = tropomyosin 1; UBE2D3 = ubiquitin-conjugating enzyme E2 D3; WFS1 = Wolfram syndrome 1. *P < 0.05 versus no ISO (n = 5 to 7 mice per group).
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Heart rate at baseline and during coronary artery occlusion and reperfusion was not different among the four experimental groups (table 2). Area at risk and myocardial infarct size are shown in figure 4. There were no significant differences ($P > 0.05$) in area at risk between C57BL/6 and miR-21 KO mice subjected to I/R injury with or without ISO treatment. Coronary occlusion followed by reperfusion resulted in an infarct size of 54±10% and 62±11% of area at risk (n = 8 mice per group) in C57BL/6 mice and miR-21 KO mice, respectively. There were no significant differences ($P > 0.05$) in infarct size between the miR-21 KO-I/R and WT-I/R groups. Pretreatment of C57BL/6 mice subjected to with ISO significantly decreased infarct size (36±10%, $P < 0.05$ vs. control, n = 8 mice per group); however, disruption of miR-21 gene abolished ISO-induced decreases in infarct size (55±9%, $P < 0.05$ vs. the ISO group, n = 8 mice per group).

**Disruption of MiR-21 Gene Abolished ISO-induced Decreases in Infarct Size**

Heart rate at baseline and during coronary artery occlusion and reperfusion was not different among the four experimental groups (table 2). Area at risk and myocardial infarct size are shown in figure 4. There were no significant differences ($P > 0.05$) in area at risk between C57BL/6 and miR-21 KO mice subjected to I/R injury with or without ISO treatment. Coronary occlusion followed by reperfusion resulted in an infarct size of 54±10% and 62±11% of area at risk (n = 8 mice per group) in C57BL/6 mice and miR-21 KO mice, respectively. There were no significant differences ($P > 0.05$) in infarct size between the miR-21 KO-I/R and WT-I/R groups. Pretreatment of C57BL/6 mice subjected to with ISO significantly decreased infarct size (36±10%, $P < 0.05$ vs. control, n = 8 mice per group); however, disruption of miR-21 gene abolished ISO-induced decreases in infarct size (55±9%, $P < 0.05$ vs. the ISO group, n = 8 mice per group).

**MiR-21 KO Abolished ISO-induced Improvements in Cardiac Function during Reperfusion**

Figure 5 demonstrates time-dependent changes in cardiac function in Langendorff-perfused hearts. Baseline values of ±dP/dt were comparable among the six groups ($P > 0.05$). There were no significant differences in ±dP/dt values between the miR-21 KO Time-C group and the WT Time-C groups ($P > 0.05$, n = 7 to 8 hearts per group) throughout the experiment. Global no-flow ischemia resulted in failure of contraction and relaxation of all hearts studied. During reperfusion, the values of ±dP/dt in all four groups of the hearts subjected to ischemia gradually increased. At 30, 60, 90, and 120 min after reperfusion, ±dP/dt were significantly greater in the

**Table 2. Heart Rate (Beats per Minute) during In Vivo Mouse Experiments**

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline</th>
<th>Coronary Occlusion</th>
<th>60 Min</th>
<th>120 Min</th>
</tr>
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<tbody>
<tr>
<td>WT-I/R</td>
<td>408±42</td>
<td>411±36</td>
<td>418±38</td>
<td>404±31</td>
</tr>
<tr>
<td>miR-21 KO-I/R</td>
<td>368±28</td>
<td>406±28</td>
<td>396±39</td>
<td>405±35</td>
</tr>
<tr>
<td>WT-I/R+ISO</td>
<td>402±34</td>
<td>419±42</td>
<td>402±32</td>
<td>399±36</td>
</tr>
<tr>
<td>miR-21 KO-I/R+ISO</td>
<td>360±42</td>
<td>375±44</td>
<td>394±31</td>
<td>413±24</td>
</tr>
</tbody>
</table>

There were no significant differences among groups (n = 8 mice per group).

I/R = ischemia-reperfusion; ISO = isoflurane; KO = knockout mice; miR-21 = microRNA-21; WT = wild-type mice.

Fig. 4. Isoflurane (ISO)-induced decreases in infarct size were blocked by the disruption of microRNA-21 (miR-21) gene. (A) Area at risk expressed as a percentage of left ventricle area; (B) myocardial infarct size expressed as a percentage of area at risk. Pentobarbital-anesthetized C57BL/6 wild-type (WT) and miR-21 knockout (KO) mice received ISO (the WT-I/R+ISO and miR-21 KO-I/R+ISO groups) or air-oxygen mixture as control (the WT-I/R and miR-21 KO-I/R groups) before 30 min of coronary artery occlusion followed by 2h of ischemia-reperfusion (I/R). The infarct area was delineated by perfusing the coronary arteries with 2,3,5-triphenyltetrazolium chloride via the aortic root, and the area at risk was delineated by perfusing phthalo blue dye into the aortic root after tying the coronary artery at the site of previous occlusion. *$P < 0.05$ versus WT-I/R and miR-21 KO-I/R; #$P < 0.05$ versus WT-I/R+ISO (n = 8 mice per group).
WT-I/R+ISO group ($P < 0.05$, $n = 9$ hearts) and depressed in the miR-21 KO-I/R group ($P < 0.05$, $n = 8$ hearts) compared with the WT-I/R group ($n = 9$ hearts). Interestingly, the values of ±dP/dt were significantly greater in the WT-I/R+ISO group than in the miR-21 KO-I/R+ISO group from 30 min to 2 h after reperfusion ($P < 0.05$, $n = 7$ to 9 hearts per group). There were no significant differences in the values of ±dP/dt at any time points between the miR-21 KO-I/R group and the miR-21 KO-I/R+ISO group ($P > 0.05$).

**MiR-21 KO Increased Mitochondrial NADH Levels during Ischemia in the Presence or Absence of ISO**

Baseline mitochondrial NADH levels from Langendorff-perfused hearts were comparable among the four experimental groups (fig. 6). During ischemia, the NADH signal initially increased and peaked 5 min after ischemia followed by a gradual decline. Peak NADH fluorescence was significantly lower in the WT-I/R+ISO group than in the WT-I/R group and greater in either the miR-21 KO-I/R or miR-21 KO-I/R+ISO group than in the WT-I/R group ($P < 0.05$, $n = 7$ to 9 hearts per group). At all time points, no significant differences existed between the miR-21 KO-I/R group and the miR-21 KO-I/R+ISO group ($P > 0.05$). During reperfusion, the NADH signal remained relatively stable in the four experimental groups. There were no significant differences in NADH fluorescence between groups during reperfusion.

**ISO Increased p-Akt, eNOS, and nNOS in C57BL/6 Hearts but Not in miR-21 KO Hearts**

The effects of ISO on Akt and p-AKT are shown in figure 7. There were no significant differences in the ratio of p-Akt/Akt between the miR-21 KO-I/R group and WT-I/R group.
**Fig. 7.** Isoflurane (ISO) increased the phosphorylation of Akt in ischemic–reperfused (I/R) myocardium of C57BL/6 wild-type (WT) mice but not in that of microRNA-21 (miR-21) knockout (KO) mice. (A) Representative Western blot bands of Akt, phosphorylated Akt (p-Akt), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a housekeeping protein. (B) Ratio of p-Akt/total Akt. C57BL/6 and miR-21 KO mice were treated, as described in figure 4. *P < 0.05 versus WT-I/R (n = 4 mice per group).

(P > 0.05, n = 4 hearts per group). Interestingly, the ratio of p-Akt/Akt was significantly increased in the WT-I/R+ISO group (P < 0.05, n = 4 hearts) but not in the miR-21 KO-I/R+ISO group (P > 0.05, n = 4 hearts) compared with the WT-I/R group. Figure 8 demonstrates the effects of ISO on the expression of eNOS and nNOS proteins. There were no significant differences in the ratio of either p-eNOS/eNOS or p-nNOS/nNOS between the miR-21 KO-I/R and WT-I/R groups. ISO significantly elevated the ratio of either p-eNOS/eNOS or p-nNOS/nNOS in C57BL/6 (P < 0.05 vs. WT-I/R, n = 4 hearts per group) but not in miR-21 KO hearts (P < 0.05 between the miR-21 KO-I/R+ISO and WT-I/R+ISO groups, n = 4 hearts per group).

**MiR-21 KO Blocked the ISO-induced Delay in the mPTP Opening**

There were no significant differences in the opening time of the mPTP between the miR-21 KO-OS and WT-OS groups (P > 0.05, n = 12 cells per group) (fig. 9). The treatment of the cardiomyocytes isolated from C57BL/6 mice with ISO delayed opening of the mPTP from 87.5 ± 7.8 s in the WT-I/R group to 103.3 ± 3.8 s in the WT-OS+ISO group (P < 0.05 between the WT-OS+ISO and WT-OS groups, n = 12 cells per group). This beneficial effect of ISO on the mPTP was eliminated by disruption of miR-21 gene (P < 0.05 between the miR-21 KO-OS+ISO and WT-OS+ISO groups and P > 0.05 between the miR-21 KO-OS+ISO and WT-OS groups).

**Regulation of Cardiomyocyte Injury by MiR-21**

The effects of pre-miR-21, anti-miR-21, and H₂O₂ on cardiomyocyte injury are shown in figure 10. GFP and pre-miR-21 did not change PI-positive cardiomyocytes compared with the control group (P > 0.05). Anti-miR-21 and OS induced by H₂O₂ increased PI-positive cardiomyocytes (P < 0.05, n = 4 hearts per group).

**Fig. 8.** Isoflurane (ISO)-induced increases in the phosphorylation of endothelial nitric oxide synthase (eNOS) and neuronal nitric oxide synthase (nNOS) were abolished by disruption of microRNA-21 (miR-21) gene. (A) Representative Western blot bands of eNOS, phosphorylated eNOS (p-eNOS), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a housekeeping protein in ischemic–reperfused (I/R) myocardium. (B) Representative Western blot bands of nNOS, phosphorylated nNOS (p-nNOS), and GAPDH as a housekeeping protein in I/R myocardium. (C) Ratio of p-eNOS/total eNOS. (D) Ratio of p-nNOS/total nNOS. C57BL/6 wild-type (WT) and miR-21 knockout (KO) mice were treated, as described in figure 4. *P < 0.05 versus WT-I/R; #P < 0.05 versus WT-I/R+ISO (n = 4 mice per group).
Discussion

The results of the current study demonstrate the involvement of miR-21 in ISO-induced protection of mouse hearts against acute I/R injury. In the WT mice, ISO up-regulates shortly the expression of the miR-21 gene, down-regulates the miR-21 target Ras homolog family member A, decreases the myocardial infarct size and the NADH levels during ischemia, improves the recovery of cardiac function after I/R injury, and increases the phosphorylation of Akt, eNOS, and nNOS in ischemic/reperfused myocardium, and delays the opening of the mPTP. These beneficial effects of ISO are lost in cardiac fibroblasts. Increased overexpression of miR-21 in cardiac fibroblasts contributes to the development of hypertrophy and fibrosis.

In the current study, the expression of cardiac miR-21 was up-regulated after ISO treatment in C57BL/6 mice. The molecular mechanisms involved in miR-21 regulation by ISO remain elusive. Accumulating evidence indicates that pretreatment of the heart and brain with ISO increases the expression of NF-κB, a key transcription factor that regulates gene programing through positive and negative feedback mechanisms. Recently, a study demonstrated that NF-κB mediated the regulation of miR-21 in cardiomyocytes. It is reasonable to speculate that the increased expression of NF-κB by ISO contributes to the up-regulation of myocardial miR-21.

MiR-21 targets multiple components of many important signal pathways, including programmed cell death 4 (PDCD4), PTEN, SPRY 1 and 2, and mitochondrial apoptosis tumor-suppressive pathways. Initially, we used qRT-PCR to analyze the expression of PDCD4, SPRY 1 and 2, and PTEN mRNAs in C57BL/6 mouse hearts after ISO treatment. There were no significant differences in the levels that cardiomyocyte-specific overexpression of miR-21 by targeted gene transfer did not impact dimensions and function of the LV. Taken together, these data suggest that alterations in cardiomyocyte miR-21 do not significantly impact overall cardiovascular function in mice. However, in heart disease, such as cardiac hypertrophy and heart failure, miR-21 appears to have a disposition to be aberrantly modified.
of PDCD4, SPRY1 and 2, and PTEN mRNAs between the ISO-treated and control groups \( (P = 0.09 \) in PTEN between the ISO and no ISO group, \( n = 7 \) \) (data not shown). Furthermore, we used mouse miFinder RT\(^2\) PCR array to analyze 84 experimentally validated and predicted targets of miR-21. \( RHOB \) was inhibited after ISO treatment. Recently, we have indicated that PDCD4 is a downstream target of miR-21 in ISO-induced protection of cardiomyocytes.\(^20\) Thus, ISO-induced up-regulation of miR-21 may alter the expression of multiple gene targets.

The pathophysiologic role of miR-21 in myocardial I/R injury is cell specific, depending on the progression of myocardial infarction.\(^3,7\) In the current study, inhibition of miR-21 with anti-miR-21 increases cell injury, and the miR-21 mimic, pre-miR-21, diminishes \( \text{H}_2\text{O}_2 \)-induced cell injury in cultured cardiomyocytes. These data are consistent with a previous study showing that increased expression of miR-21 in cardiac myocytes reduces myocardial I/R injury in the early phase of myocardial infarction.\(^7\) However, in the late phase of myocardial infarction, miR-21 is overexpressed predominantly in cardiac fibroblasts, that is associated with fibrosis and cardiac remodeling.\(^32\) To examine the dependence of ISO-induced cardioprotection on miR-21, we used miR-21 KO mice. Myocardial infarct size was comparable between miR-21 KO mice and WT mice subjected to a 30-min coronary occlusion followed by a 2-h reperfusion. Nonetheless, myocardial infarct size was significantly decreased by ISO in WT mice but not in miR-21 KO mice. Furthermore, cardiac function during reperfusion was significantly improved by ISO in isolated C57BL/6 hearts but not in miR-21 KO hearts. These \textit{in vivo} and \textit{ex vivo} data indicate that miR-21 is involved in cardioprotection against acute I/R injury by ISO.

Nicotinamide adenine dinucleotide is a substrate for respiratory complex I in the electron transport chain in mitochondria. It can emit fluorescence when mitochondria are illuminated at the appropriate wavelength. Under physiologic conditions, NADH levels remain stable by the balance between its generation (mitochondrial dehydrogenase) and its consumption (electron transfer). During myocardial ischemia/hypoxia, NADH fluorescence is significantly increased mainly due to dysfunction of electron transfer in mitochondria.\(^26\) During the present investigation, ischemia-induced increases in NADH signal were consistent with alterations in mitochondrial homeostasis regulate cardiac tolerance to I/R injury.\(^2,35\) In the current study, ISO increased phosphorylation of both eNOS and nNOS, and this action was blocked by disruption of miR-21 gene. In contrast, phosphorylated glycerone synthase kinase 3β and Pim-1 were not altered by disruption of miR-21 gene (data not shown). Thus, both eNOS and nNOS appear to be the downstream targets of Akt in the ISO-induced cardioprotection against I/R injury.

The mPTP plays a crucial role in myocardial I/R injury and protection.\(^38,39\) In the current study, we measured time of the mPTP opening in cardiomyocytes isolated from adult mice. Opening of the mPTP induced by photoexcitation-generated OS was delayed by ISO in cardiomyocytes isolated from WT mice. This confirms our previous findings in cardiomyocytes isolated from rat hearts.\(^28,39\) Interestingly, the protective effects of ISO on mitochondria were abolished in the miR-21 KO mice, suggesting that mitochondria are a downstream effector of miR-21. The signaling pathway linking miR-21 to the mPTP remains elusive. Previously, we indicate that eNOS regulates function of mPTP at early reperfusion in mice.\(^39\) Given that disruption of miR-21 gene blocked ISO-induced phosphorylation of both eNOS and nNOS, nitric oxide generated by eNOS and nNOS may be involved in the modulation of the mPTP opening.\(^30\)

One limitation of this study is that there are no array-based analyses of microRNAs in mouse hearts after the treatment of ISO. In rat hearts or neonatal rat cardiomyocytes, our array analyses of microRNAs indicate that ISO up-regulated 11 of 87 microRNAs studied, including miR-21, microRNA-210, and microRNA-30 family.\(^26\) It is likely that multiple microRNAs are involved in the cardioprotecive effect of ISO. However, miR-21 plays an important role in protection of cardiomyocytes against hypoxia/reoxygenation injury.\(^20\) Another limitation is that this study does not involve the long-term outcome of miR-21 up-regulation by ISO because overexpression of miR-21 in fibroblasts is associated with hypertrophy and fibrosis in diseased state.\(^10,16\) However, ISO-induced increase in miR-21 mRNA lasted...
approximately 6h after ISO exposure. It is reasonably believed that this temporary up-regulation of miR-21 is not sufficient to cause significant hypertrophy and fibrosis.

In summary, the current study demonstrates the pivotal role of miR-21 in ISO-induced cardioprotection against acute I/R injury. The findings reveal new mechanisms involved in miR-21-induced myocardial protection. As a main “cardiac” microRNA, miR-21 is up-modulated in cardiomyocytes in the early phase of myocardial infarction, which contributes to myocardial protection. However, in the late phase of myocardial infarction, miR-21 is overexpressed predominantly in fibroblasts, which causes fibrosis and cardiac remodeling. Because ISO up-regulates the expression of miR-21 in myocardium, future studies will examine the effect of ISO on cardiac miR-21 and the contribution of miR-21 modulation to cardiac fibrosis and remodeling after myocardial infarction.

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Competing Interests
The authors declare no competing interests.

Correspondence
Address correspondence to Dr. Ge: Department of Anesthesiology, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, Wisconsin 53226. zdge@mcw.edu. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. ANESTHESIOLOGY’s articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

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