Therapeutic administration of IL-11 exhibits the postconditioning effects against ischemia-reperfusion injury via STAT3 in the heart

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ISCHEMIA-REPERFUSION (I/R) is one of the major causes of myocardial injury in the clinical setting, especially in the therapeutic process of acute myocardial infarction. Although various kinds of preventive therapies from I/R injury have been proposed so far, clinical trials revealed that they are insufficient. Therefore, it is urgent to develop the therapeutic strategy on a novel concept to prevent myocardial damage after I/R. Accumulating evidence has shown that cardiac homeostasis is maintained by a wide range of neurohumoral factors and cytokines, suggesting that these factors could be therapeutic targets for cardioprotection.

IL-6 family cytokines contribute to cardioprotection by activating various kinds of signaling molecules. In their cytokine signaling pathways, activation of glycoprotein 130/STAT3 axis plays important roles in cytoprotection and angiogenesis (6, 21, 22). Thus the activation of STAT3 by IL-6 family cytokines is considered a potential therapeutic strategy for cardiovascular diseases (5). Experimentally, leukemia inhibitory factor shows the antifibrotic effect after myocardial infarction (29); however, this cytokine has not been considered clinically appropriate because of its proinflammatory properties (7). To establish a novel therapeutic strategy against cardiovascular disease, we focused on IL-11, a member of IL-6 family cytokines, because its proinflammatory activity is limited and because IL-11 exhibits anti-inflammatory activity in some cases (3, 23).

IL-11 exhibits multipotential functions (4). Because IL-11 has the thrombopoietic activity, recombinant human IL-11 is clinically used for thrombocytopenia (9). In addition, IL-11 also shows nonhematopoietic functions. Previously, we reported that the therapeutic treatment of IL-11 reduces adverse cardiac remodeling after myocardial infarction in murine model, concomitant with anti-apoptosis and angiogenesis (20). Furthermore, cardiac-specific ablation of STAT3 abrogated IL-11-mediated attenuation of adverse cardiac remodeling, suggesting that cardiac activation of STAT3 mediates anti-fibrotic effects.

In this study, to address the possibility of clinical application of IL-11 treatment in the therapeutic process of acute myocardial infarction as a cardioprotective strategy, we investigated its postconditioning effects on I/R injury. In addition, we examined whether IL-11 utilizes the cardiac STAT3 signaling pathway in its postconditioning effects.

MATERIALS AND METHODS

Animal care. The care of all animals was approved by the Animal Care and Use Committee of Graduate School of Pharmaceutical Sciences, Osaka University. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the
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National Institutes of Health (NIH publication No. 85-23, revised 1996).

All mice for the experiments were euthanized by inhalation of isoflurane in a euthanasia chamber. Death of the animals was confirmed by monitoring the absence of breath after removal of the carcass from the euthanasia chamber. A total of 172 mice were used in this study.

**I/R model and IL-11 treatment.** Murine I/R was generated as described previously, with minor modifications (15, 21). Briefly, C57BL/6 mice (8- to 12 wk old; Japan SLC) were anesthetized and ventilated with 80% oxygen containing 1.5% isoflurane (Merck). After left-side thoracotomy, 7-0 silk suture was tied around the left coronary artery with a slipknot. Infarction was confirmed by discoloration of the ventricle and ST-T changes in electrocardiogram monitor. The chest and the skin were closed with 5-0 silk sutures. The mice were revived for a 30-min ischemic period, after which the knot was released and the heart was allowed to reperfuse for 24 h. By this experimental protocol, the mortality was minimized to less than 10%. Twenty four hours after reperfusion, the mice were euthanized and the slipknot was retied. PBS containing 1.5% Evans blue was injected retrogradely. Isolated hearts were sectioned, and viable myocardium was stained with 2% triphenyl tetrazolium chloride (Sigma), as described previously (21). The amounts of myocardial area not at risk, area at risk (AAR), and infarcted area were quantified with Scion Image (Scion). In the IL-11 group, basically, 20 μg/kg of recombinant human IL-11 (Peprotech) was intravenously administered at the start of reperfusion (various concentrations in 200 μl of PBS/25 g of body wt), whereas the control group received the same volume of PBS over the same period. In the study concerning the dose-dependent effects of IL-11 on myocardial injury, various concentrations (3, 8, 20, 50 μg/kg) of IL-11 were used. There was no difference in mortality between groups.

**Immunoblot analysis.** Immunoblot analyses were performed as described previously (18). Briefly, heart homogenates were prepared in buffer containing 150 mM NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1% Triton X-100, 1% deoxycholic acid, 1% protease inhibitor cocktail, 1 mM dithiothreitol, 1 mM sodium orthovanadate, and 1 mM NaF. Proteins were separated by SDS-PAGE and transferred onto the polyvinylidene difluoride membrane (Millipore). The membrane was immunoblotted with anti-phospho-STAT3 (p-STAT3; Cell Signaling Technology) or anti-STAT3 (Santa Cruz Biotechnology) antibody. The membrane was reprobed with anti-STAT3 or anti-GAPDH (Santa Cruz Biotechnology) antibody to show the equal amount loading. Electrochemiluminescence system was used for the detection.

**Echocardiographic analysis.** Mice were exposed to I/R injury and IL-11 (20 μg/kg) or PBS, as a control, was administered at start of reperfusion. Twenty four hours after reperfusion, two-dimensional and motionmode (M-mode) transthoracic echocardiography was performed using an iE33 model equipped with a 15-MHz transducer (Philips Electronics, Andover, MA). Echocardiographic measurements were taken on M-mode. The investigator was blinded to the identity of the mice for analysis. Sham indicates the mice underwent thoracotomy without I/R.

**Hemodynamic analysis.** Hemodynamics was analyzed according to previous report with minor modification (20). Briefly, 24 h after reperfusion, mice were anesthetized (50 mg/kg pentobarbital) and heparinized (50 units) via intraperitoneal injection. The hearts were rapidly excised and placed in ice-cold modified Tyrode’s solution containing (in mM) 140 NaCl, 5.4 KCl, 1.8 CaCl₂, 0.45 MgCl₂, 0.33 NaH₂PO₄, 5.5 glucose, and 5 HEPES (pH 7.4). The aorta was cannulated and retrogradely perfused at a constant pressure of 100 mmH₂O with Tyrode’s solution bubbled with 80% oxygen at 37°C. Thus the experiments were performed at 37°C by immersing the heart in Tyrode’s solution in a water-jacketed chamber. The hearts were paced at 420 beats/min. The fluid-filled balloon was inserted into the left ventricle to monitor cardiac function. The balloon was attached to a pressure transducer, which was coupled to a 4S PowerLab (AD Instruments). Left ventricular developed pressure and maximal and minimal change in pressure over time were measured.

**Terminal deoxynucleotide transferase-mediated dUTP nick-end labeling staining.** Twenty four hours after reperfusion, the frozen sections (5 μm thick) were prepared from the portion in the middle of the infarct zone. Apoptotic cell death was detected by terminal deoxynucleotide transferase-mediated dUTP nick-end labeling (TUNEL) staining with in situ apoptosis detection kit (TaKaRa). The sections were costained with anti-sarcomeric α-actinin (Sigma) antibody to identify the cardiomyocytes. Nuclei were simultaneously stained with Hoechst 33258. For quantitative analyses, apoptotic myocytes were counted in number by the researcher who was blinded to the assay conditions.

Fig. 1. IL-11 treatment enhanced STAT3 activation in ischemia/reperfusion (I/R) hearts. A: mice were exposed to I/R. At indicated time points, mice were euthanized and the lysates from hearts were immunoblotted with anti-phospho-specific STAT3 (p-STAT3) antibody. The blots were reprobed with anti-STAT3 antibody or GAPDH antibody. Representative data (top) and quantitative analyses of the p-STAT3 (bottom) are shown. Data are shown as means ± SD (n = 3 mice for each condition). *P < 0.05 vs. nonoperation, by 1-way ANOVA followed by Bonferroni test. B: mice were exposed to I/R. IL-11 (20 μg/kg) or PBS, as a control, was administered intravenously at start of reperfusion. Fifteen minutes after treatment, mice were euthanized and the lysates from hearts were immunoblotted with anti-p-STAT3 antibody. Blots were reprobed with anti-STAT3 antibody or GAPDH antibody. Representative data (top) and quantitative analyses of the p-STAT3 (bottom) are shown. Data are shown as means ± SD (n = 4 mice for each condition). *P < 0.05 vs. nonoperation, by 1-way ANOVA followed by Bonferroni test.
CTT GCA GGA AG; cyclooxygenase (COX)-2, forward: ACT GCC CAA CTC CCA TGG GT, reverse: AGT CCA CTC CAT GGC CCA GT; MnSOD, forward: AGG ACG GCA GCG GTT TGG TAA ACC T, reverse: CGG TGG CTG TGA TGT TGG TCA CGT A; Cu/ZnSOD, forward: AGA GCC TGA CAG GTG CAG AGA ACC, reverse: ACT TTG GCA TGG GTG TCG CC; redox factor-1, forward: AGA GAC CAA GAG GAG TAA GGG G, reverse: TGC TTC GTT TTA TCC TGC; 5-oxoprolinase, forward: TTC CAG TCC AGT C, reverse: CTT CCA TGG GT; nuclear factor-like 1, forward: TGC ACA GTT CCC G, reverse: TCT GTG GAT GTG CCT GT; glutathione isocitrate dehydrogenase, forward: AAG GAG AAG CTC ATC CTG T, reverse: TAA GCA TTG ATG TCC TTG CTC CAG; peroxiredoxin 5, forward: TCA GCT TGA ACT CTT CCA CAC G, reverse: TGC TTC GTT TTA TCC TGC; peroxiredoxin 6, forward: AGA TTC ATG GGG CAT TCT CTT C, reverse: TAA GCA TTG ATG TCC TTG CTC CAG; redox factor-1, forward: AGT TGG GAG ATG AGT TAC TGA G, reverse: CAT CCG TCT GAA TGC CCA CT; glutathione peroxidase 4, forward: AGG CAG GAC CCA GGA AGT A, reverse: TGA TGG CAT TTC CCA GCA TGG; 5-oxoprolinase, forward: TGG CAG CTA AAG AAT G, reverse: TCT GTG GAT GTG CCT CCC ATG T; nuclear factor-like 1, forward: TGA ACA GAT CCC AGC TGA C, reverse: CTT CCA TAG CCT GCA TTT CCA T;

**Table 1. Effects of IL-11 on cardiac function at 24 h after reperfusion**

<table>
<thead>
<tr>
<th>Parameter/Group</th>
<th>Sham</th>
<th>I/R + PBS</th>
<th>I/R + IL-11</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ejection fraction, %</td>
<td>79.3 ± 2.5</td>
<td>52.2 ± 5.7*</td>
<td>62.9 ± 10.1#*</td>
</tr>
<tr>
<td>Fractional shortening, %</td>
<td>40.8 ± 1.9</td>
<td>21.9 ± 3.1*</td>
<td>28.6 ± 6.2#*</td>
</tr>
<tr>
<td>Diastolic interventricular septal thickness, cm</td>
<td>0.085 ± 0.001</td>
<td>0.085 ± 0.012</td>
<td>0.093 ± 0.005</td>
</tr>
<tr>
<td>LV, cm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diastolic internal diameter</td>
<td>0.391 ± 0.033</td>
<td>0.360 ± 0.036</td>
<td>0.378 ± 0.009</td>
</tr>
<tr>
<td>Diastolic posterior wall thickness</td>
<td>0.069 ± 0.003</td>
<td>0.080 ± 0.012</td>
<td>0.077 ± 0.011</td>
</tr>
<tr>
<td>Systolic internal diameter</td>
<td>0.232 ± 0.026</td>
<td>0.282 ± 0.035</td>
<td>0.270 ± 0.028</td>
</tr>
<tr>
<td>Heart rate-LV, beats/min</td>
<td>494 ± 21</td>
<td>490 ± 52</td>
<td>486 ± 45</td>
</tr>
</tbody>
</table>

Values are means ± SD; *n = 3 mice for sham; n = 6 mice for ischemia-reperfusion (I/R) + PBS; and n = 6 mice for I/R + IL-11. Mice were subjected to 30 min of ischemia followed by 24 h reperfusion. IL-11 (20 μg/kg) or PBS, as a control, was intravenously administered at the time of reperfusion. $P < 0.01; $#P < 0.05 vs. Sham; *$P < 0.05 vs. I/R + PBS, by unpaired t-test. LV, left ventricular.
Table 2. Effects of IL-11 on hemodynamics at 24 h after reperfusion

<table>
<thead>
<tr>
<th>Parameter/Group</th>
<th>Sham</th>
<th>I/R + PBS</th>
<th>I/R + IL-11</th>
</tr>
</thead>
<tbody>
<tr>
<td>LV developed pressure, mmHg</td>
<td>79.5 ± 9.9</td>
<td>55.6 ± 11.05</td>
<td>69.3 ± 6.7*</td>
</tr>
<tr>
<td>+dP/dt (mmHg/s)</td>
<td>2347.8 ± 443.2</td>
<td>1532.4 ± 296.15</td>
<td>2090.0 ± 321.2*</td>
</tr>
<tr>
<td>−dP/dt (mmHg/s)</td>
<td>−2178.3 ± 387.5</td>
<td>−1395.0 ± 326.18</td>
<td>−1776.7 ± 132.25*</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = 4 mice for sham, n = 5 mice for I/R + PBS, and n = 6 mice for I/R + IL-11. Mice were subjected to 30 min of ischemia followed by 24 h reperfusion. IL-11 (20 μg/kg) or PBS, as a control, was intravenously administered at the time of reperfusion. $P < 0.05$ vs. Sham; *$P < 0.05$ vs. I/R + PBS, by unpaired t-test. ±dP/dt: maximal and minimal change in pressure over time.

GAPDH, forward: GCC GGT GCT GAG TAT GTC GT, reverse: CCC TTT TGG CTC CAC CCT T.

Cell culture and reagents. Cardiomyocytes were cultured as described previously (16). Briefly, cardiac ventricles of 1-day-old Wistar rats were minced and cells were isolated with 0.1% trypsin (Difco Laboratories) and 0.1% collagenase type IV (Sigma). To eliminate the nonmyocyte population, isolated cells were plated and incubated for 1 h at 37°C. Nonattached cells were collected as cardiomyocytes and cultured in DMEM/Ham’s F-12 (DMEM/F-12) containing 5% neonatal calf serum. More than 90% of cells were identified as cardiomyocytes, assessed by immunostaining with anti-sarcomeric specific α-actinin antibody.

STAT3 Stealth RNAi, MT Stealth RNAi, and control Stealth RNAi were purchased from Invitrogen. Cardiomyocytes were transfected with these small interfering RNA (siRNA) using Lipofectamine RNAiMAX (Invitrogen) in DMEM/F-12 containing 5% neonatal calf serum. Cardiomyocytes were cultured in serum-free DMEM/F-12 containing IL-11 and/or H2O2 at the indicated concentrations. Apoptotic cells were detected by Annexin V staining, as described in a previous report (16).

Conditional ablation of STAT3 gene in cardiomyocytes of adult murine hearts. Cardiac STAT3 conditional knockout mice were generated as described previously with minor modifications (20). In brief, the cardiac-specific transgenic mice overexpressing Cre recombinase fusion protein to the mutated estrogen receptor domains (MerCreMer) under the control of α-myosin heavy chain (α-MHC) promoter were crossed with STAT3 flox mice (STAT3floxplox) to produce α-MHC-MerCreMer/STAT3floxplox mice. To activate Cre-recombinase activity, α-MHC-MerCreMer/STAT3floxplox or α-MHC-MerCreMer/STAT3wild/wild mice, as control mice, were intraperitoneally injected with 8 mg/kg of tamoxifen (Sigma) dissolved in corn oil (Sigma) once a day for 14 consecutive days. After tamoxifen treatment, the mutant mice underwent I/R as described above.

Statistical analysis. Data were presented as means ± SD. The comparison between two groups was performed using an unpaired t-test. One-way ANOVA with Bonferroni test was used for comparisons of multiple groups. Differences were considered statistically significant when the calculated P value was less than 0.05.

RESULTS

IL-11 treatment enhanced STAT3 activity in I/R hearts. Because it is known that STAT3 is activated during I/R, we first confirmed that STAT3 is endogenously activated in hearts at various time points after I/R in our system (Fig. 1A). Immunoblot analyses with anti-p-STAT3 antibody revealed that STAT3 phosphorylation was slightly induced at a 30-min ischemia period. It is important that STAT3 was dramatically activated at 1 h after reperfusion. These data indicated that STAT3 signals are endogenously activated during I/R.

Next, we examined whether IL-11 administration further enhanced STAT3 activity during I/R (Fig. 1B). Mice were exposed to I/R injury with intravenous injection of IL-11 at a dose of 20 μg/kg or PBS at start of reperfusion. We analyzed the activation of STAT3 at 15 min after reperfusion by immunoblot analysis, based on the previous findings that the intravenous administration of IL-11 activated STAT3 with its peak at 15 min in nonoperated hearts (20). Fifteen minutes after reperfusion, I/R stimuli induced STAT3 activation, which was enhanced by IL-11 treatment, relative to control.

The therapeutic treatment of IL-11 exhibits the postconditioning effects against I/R injury. Because STAT3 activity was reinforced by IL-11 injection at start of reperfusion in I/R...
model, we examined the postconditioning effects of IL-11 on I/R injury (Fig. 2). Mice were subjected to 30 min of left coronary artery ligation followed by 24 h reperfusion. IL-11 or PBS, as a control, was administered intravenously at start of reperfusion. Although there was no significant difference in AAR between IL-11 treatment and control group, the infarct size relative to AAR was decreased by single treatment of IL-11 in a dose-dependent manner (Fig. 2C). Treatment of IL-11 at 20 and 50 μg/kg significantly reduced the infarct size by 38.8 and 39.2%, respectively (PBS, 46.7 ± 14.4%; 20 μg/kg of IL-11, 28.6 ± 7.5%; 50 μg/kg of IL-11, 28.4 ± 13.7%). Because it is confirmed that IL-11 treatment at a dose of 20 μg/kg achieved the maximal effect, further studies were performed with the use of IL-11 at a dose of 20 μg/kg. Next, we investigated the cardiac function after I/R by echocardiographic and hemodynamic analysis. Echocardiographic analysis revealed that ejection fraction and fraction shortening were dramatically reduced 24 h after reperfusion (Table 1). Furthermore, hemodynamic analysis elucidated that left ventricular developed pressure and maximal and minimal change in pressure over time were reduced by I/R (Table 2). It is intriguing, however, that IL-11 treatment at a dose of 20 μg/kg significantly preserved cardiac function.

We also examined the effects of the timing of IL-11 treatment on cardioprotection. Mice were exposed to I/R injury, and IL-11 was treated at 3 h after reperfusion. As a result, IL-11 was less effective in cardioprotection when administered at 3 h than immediately after reperfusion (data not shown). Therefore, the therapeutic window of IL-11 is likely to be the early time point after myocardial infarction.

These findings suggest that the administration of IL-11 at reperfusion has the therapeutic potential to prevent I/R injury.

Table 3. The expressions of cytoprotective genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Non-IL-11 + PBS</th>
<th>I/R + PBS</th>
<th>I/R + IL-11</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metallothionein 1</td>
<td>6.73 ± 1.57*</td>
<td>9.48 ± 2.15*</td>
<td>13.61 ± 3.04* #</td>
</tr>
<tr>
<td>Metallothionein 2</td>
<td>4.07 ± 0.82*</td>
<td>9.04 ± 2.97*</td>
<td>13.58 ± 2.63* #</td>
</tr>
<tr>
<td>Cyclooxygenase-2</td>
<td>0.41 ± 0.20</td>
<td>16.85 ± 10.61*</td>
<td>17.86 ± 2.83*</td>
</tr>
<tr>
<td>MnSOD</td>
<td>1.09 ± 0.08</td>
<td>1.12 ± 0.15</td>
<td>1.09 ± 0.17</td>
</tr>
<tr>
<td>Cu/ZnSOD</td>
<td>1.09 ± 0.12</td>
<td>0.95 ± 0.08</td>
<td>1.09 ± 0.22</td>
</tr>
<tr>
<td>Redox factor-1</td>
<td>2.77 ± 3.07</td>
<td>3.76 ± 5.86</td>
<td>1.73 ± 0.24*</td>
</tr>
<tr>
<td>Peroxiredoxin 5</td>
<td>1.02 ± 0.12</td>
<td>1.12 ± 0.21</td>
<td>1.09 ± 0.23</td>
</tr>
<tr>
<td>Peroxiredoxin 6</td>
<td>1.06 ± 0.11</td>
<td>1.10 ± 0.20</td>
<td>1.15 ± 0.29</td>
</tr>
<tr>
<td>Isocitrate dehydrogenase</td>
<td>1.08 ± 0.04</td>
<td>1.18 ± 0.17</td>
<td>1.15 ± 0.29</td>
</tr>
<tr>
<td>Glutathione reductase</td>
<td>0.68 ± 0.29</td>
<td>1.12 ± 0.67</td>
<td>1.48 ± 0.81</td>
</tr>
<tr>
<td>Glutathione peroxidase 4</td>
<td>1.03 ± 0.12</td>
<td>1.11 ± 0.19</td>
<td>1.12 ± 0.28</td>
</tr>
<tr>
<td>5-Oxoprolinase</td>
<td>1.52 ± 0.45</td>
<td>1.11 ± 0.39</td>
<td>1.18 ± 0.31</td>
</tr>
<tr>
<td>Nuclear factor-like 1</td>
<td>1.28 ± 0.04</td>
<td>1.20 ± 0.14</td>
<td>1.29 ± 0.24</td>
</tr>
</tbody>
</table>

Values (fold change of control mice) are means ± SD; n = 3–7 mice. Three hours after treatment of IL-11 at 20 μg/kg or PBS, total RNA was prepared from I/R or nonoperation hearts and real time RT-PCR was performed for the cytoprotective genes. The expression of cytoprotective genes was normalized with that of GAPDH. *P < 0.05 vs. control mice; #P < 0.05 vs. I/R + PBS, by unpaired t-test.
IL-11 treatment suppressed apoptotic cell death and reactive oxygen species generation in I/R hearts. To address the mechanisms of the postconditioning effects of IL-11, we examined whether IL-11 treatment prevented apoptotic cell death by I/R injury (Fig. 3). TUNEL staining revealed that TUNEL-positive cardiomyocytes were detected mainly at the risk area 24 h after reperfusion. It is important that IL-11 treatment significantly reduced the frequency of apoptotic cardiomyocytes compared with the PBS group.

Reactive oxygen species (ROS) is one of the most important inducers of apoptotic and necrotic cell death after I/R. To assess the mechanism of IL-11-mediated suppression of cell death, we focused on the effect of IL-11 on ROS production (Fig. 4A). DHE fluorescence staining demonstrated that ROS was induced by I/R at risk area. It is interesting that IL-11 treatment suppressed ROS production relative to PBS group.

To clarify the molecular mechanism of the ROS-scavenging effects of IL-11, the expression of ROS-related cytoprotective genes downstream of STAT3 (19, 21, 24, 27) was measured by real time RT-PCR (Table 3). Among them, MT1, MT2, and COX-2 mRNA were upregulated at 3 h after I/R. It is important that IL-11 treatment enhanced the I/R-induced upregulation of MT1 and MT2 gene transcripts at 3 h after reperfusion (Fig. 4B), whereas the expression of COX-2 mRNA was not reinforced by IL-11 under I/R condition.

We examined whether IL-11 induced MT expression through STAT3 activation in vitro and in vivo (Fig. 5). In the in vitro model, neonatal rat cardiomyocytes were prepared and transfected with control siRNA or with STAT3 siRNA. Twenty four hours later, cells were stimulated with IL-11 (20 ng/ml) for 3 h. Real-time PCR analyses revealed that the stimulation with IL-11 resulted in the increased expressions of MT1 and MT2, which was cancelled by the knockdown of STAT3 in the in vivo model.

MT1 and MT2 expression was significantly enhanced by IL-11 treatment compared with the PBS group. In contrast, COX-2 expression was not enhanced by IL-11 treatment. We examined whether IL-11 induced MT expression through STAT3 activation in vitro and in vivo (Fig. 5). In the in vitro model, neonatal rat cardiomyocytes were prepared and transfected with control small interfering RNA (siRNA; control) or with STAT3 siRNA (STAT3). Twenty four hours later, cells were stimulated with IL-11 (20 ng/ml) for 3 h. Real-time PCR analyses revealed that the stimulation with IL-11 resulted in the increased expressions of MT1 and MT2, which was cancelled by the knockdown of STAT3. In the in vivo model, STAT3 gene was ablated in a cardiomyocyte-specific manner, using cardiomyocyte-specific tamoxifen-inducible Cre recombinase transgenic mice (α-
MHC-MerCreMer mice) on the genetic background of STAT3\textsuperscript{ox/ox}. \(\alpha\)-MHC-MerCreMer mice on STAT3\textsuperscript{wild/wild} were used as a control. To induce Cre-mediated recombination, mice were treated with tamoxifen as described in MATERIALS AND METHODS. After tamoxifen treatment, the level of STAT3 protein expression decreased (data not shown). Cardiac-specific conditional STAT3-deficient (STAT3 CKO) mice and control (Wild) mice were administered with IL-11. Three hours after IL-11 administration, MT1 and MT2 mRNA expressions were measured by real-time PCR. Although IL-11 increased MT1 and MT2 mRNA in Wild mice, the upregulation of MT1 and MT2 mRNA by IL-11 was abrogated in STAT3 CKO mice.

To address the importance of MT1 and MT2 in IL-11-mediated cardioprotection, cardiomyocytes were transfected with control siRNA or with MT1 and 2 siRNA. Twenty four hours later, cells were incubated with IL-11 (200 ng/ml) for 6 h, followed by exposure to \(\text{H}_2\text{O}_2\) (1 mM) for 24 h. Apoptotic cells were detected by Annexin V staining. Although \(\text{H}_2\text{O}_2\) evoked apoptosis in control siRNA-transfected cardiomyocytes, IL-11 suppressed \(\text{H}_2\text{O}_2\)-induced apoptosis, as reported previously (16). It is important that IL-11-mediated attenuation of \(\text{H}_2\text{O}_2\)-induced apoptosis was reduced in MT siRNA-transfected cardiomyocytes. These data indicated that IL-11 prevented ROS-induced cardiomyocyte death, at least partially through STAT3/MIT axis.

**DISCUSSION**

In the present study, we examined the postconditioning effects of therapeutic treatment of IL-11 on I/R injury. IL-11 administration enhanced the I/R-induced activation of STAT3 in hearts. Single treatment of IL-11 at the start of reperfusion was sufficient to attenuate I/R injury in mice. IL-11 prevented apoptotic cell death and ROS generation, accompanied by the increase of MT1 and 2 mRNA expressions. It is important that IL-11-mediated cardioprotection against I/R injury was abolished in cardiac-specific conditional STAT3-deficient mice.

![Fig. 6. IL-11-mediated postconditioning against I/R injury was abrogated in STAT3 CKO mice. \(\alpha\)-Myosin heavy chain (\(\alpha\)-MHC) MerCreMer/STAT3\textsuperscript{ox/ox} (CKO) mice or \(\alpha\)-MHC MerCreMer STAT3\textsuperscript{wild/wild} (Wild) mice, as control mice, were intraperitoneally administered with 8 mg/kg of tamoxifen once a day for 14 consecutive days. After tamoxifen treatment, STAT3 CKO mice and wild-type mice were subjected to 30 min ischemia, followed by 24 h reperfusion. IL-11 (20 \(\mu\)g/kg) was administrated intravenously at start of reperfusion. A and B: areas at risk were estimated by exclusion of Evans blue. The myocardial infarct areas were detected by staining with 2% TTC. Risk area size and infarct size were quantitatively estimated. Data are shown as means ± SD (n = 7 mice for Wild-PBS; n = 5 mice for Wild-IL-11; n = 8 mice for CKO-PBS; n = 6 mice for CKO-IL-11). *P < 0.05 by unpaired t-test. C: twenty four hours after reperfusion, the heart sections were stained with DHE. The fluorescence intensity at risk area was estimated. Data are shown as means ± SD (n = 4 mice for Wild-PBS; n = 6 mice for Wild-IL-11; n = 6 mice for CKO-IL-11). *P < 0.05 by unpaired t-test; NS, not significant.](http://ajpheart.physiology.org/doi/10.1152/ajpheart.00060.2012)
Furthermore, IL-11 treatment failed to suppress ROS generation in response to I/R in STAT3 CKO mice. These findings suggest that IL-11 treatment exhibits postconditioning effects through STAT3 pathway in I/R.

IL-11 treatment exhibits cardioprotective effects by distinct mechanisms from the cytokine therapies proposed so far. Recently, much attention has been paid to the cardioprotective effects of granulocyte colony stimulating factor (G-CSF). The administration of G-CSF preserves myocardium in the myocardial infarction model (11) as well as the I/R model (26). In G-CSF signaling, JAK2/STAT3 pathway has been proposed to be important in antifibrotic effects after myocardial infarction during subacute and chronic phase, whereas the Akt/NOS pathway contributes to the beneficial effect of G-CSF in I/R hearts during the acute phase (25). In contrast, IL-11 protects myocardium through STAT3 both in acute and chronic phase. IL-11 attenuated adverse cardiac remodeling after myocardial infarction via cardiac STAT3 pathway (20). And, herein, we revealed that IL-11-mediated suppression of I/R injury was exerted by cardiac STAT3 activation in acute phase. We have also confirmed that IL-11 does not activate Akt signal in murine hearts and neonatal rat cardiomyocytes (data not shown). Thus IL-11 therapy against I/R injury is based on a novel concept, mainly utilizing STAT3 signal.

In the present study, cardiac STAT3 activation is a critical event for IL-11-mediated postconditioning effects in I/R injury. Consistent with our results, previous studies have demonstrated that STAT3 is indispensable for ischemic postconditioning that was induced by repeated exposure to ischemic stress (1). Ischemic postconditioning is complicated and requires at least three signaling pathways, including STAT3, Akt, and TNF-α (8, 17). Indeed, the deficiency in any one of these three signaling pathways abrogates ischemic postconditioning effects; however, it has not been addressed whether the activation of one of these signals could confer the resistance to I/R under the postconditioning situation. Here, as a novel pharmacological approach, we have proposed that the activation of glycoprotein 130/STAT3 pathway simply by IL-11 administration is therapeutically sufficient for postconditioning effects.

It is important that IL-11-mediated suppression of ROS generation was abrogated in STAT3 CKO mice, suggesting that STAT3 utilizes ROS scavenging systems in its cardioprotection. It is interesting that I/R led to the increase of MT1 and 2 expressions, which were enhanced by IL-11. Combined with the previous study that MT1 and 2 are key molecules in cardiac STAT3-mediated attenuation of I/R injury (21), we consider that IL-11 prevented I/R injury at least partially through elevation of MT expression; however, we cannot exclude the possibility that other functions may be involved in STAT3-mediated cardioprotection. For example, recent studies demonstrated that STAT3 exerts protective functions, independently of its transcriptional activation (10). STAT3 contributes to cardioprotection by stimulation of respiration and inhibition of mitochondrial permeability transition pore opening in mitochondria (2). Further investigation might be needed to fully reveal the comprehensive mechanisms of postconditioning effects by IL-11/cardiac STAT3 axis.

In our experiments, IL-11 was intravenously administered to mice immediately after reperfusion to protect against cardiac injury. To utilize IL-11 in clinical situation, it might be informative to determine the therapeutic window of the IL-11 postconditioning. According to previous reports regarding G-CSF, its beneficial effects were exerted when its treatment was started early after myocardial infarction in animal models (11) and clinical trials (13, 28). Because IL-11 directly transduces cardioprotective signals in cardiomyocytes exposed to I/R stress, as is the case with G-CSF, the maximal effects of IL-11 would be achieved by its administration at the early time point of myocardial infarction.

In conclusion, IL-11 exhibits the postconditioning effects against I/R via cardiac STAT3 pathway. Our results propose the IL-11/STAT3 axis is a promising therapeutic target against ischemic heart diseases.


