Interleukin-6 mediates exercise preconditioning against myocardial ischemia reperfusion injury

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Submitted 25 November 2014; accepted in final form 23 March 2015

Interleukin-6 mediates exercise preconditioning against myocardial ischemia reperfusion injury. Am J Physiol Heart Circ Physiol 308: H1423–H1433, 2015. First published March 27, 2015; doi:10.1152/ajpheart.00850.2014.—Interleukin-6 (IL-6) is a pleiotropic cytokine that protects against cardiac ischemia-reperfusion (I/R) injury following pharmacological and ischemic preconditioning (IPC), but the affiliated role in exercise preconditioning is unknown. Our study purpose was to characterize exercise-induced IL-6 cardiac signaling (aim 1) and evaluate myocardial preconditioning (aim 2). In aim 1, C57 and IL-6−/− mice underwent 3 days of treadmill exercise for 60 min/day at 18 m/min. Serum, gastrocnemius, and heart were collected preexercise, immediately postexercise, and 30 and 60 min following the final exercise session and analyzed for indexes of IL-6 signaling. For aim 2, a separate cohort of exercise-preconditioned (C57 EX and IL-6−/− EX) and sedentary (C57 SED and IL-6−/− SED) mice received surgical I/R injury (30 min I, 120 min R) or a time-matched sham operation. Ischemic and perfused tissues were examined for necrosis, apoptosis, and autophagy. In aim 1, serum IL-6 and IL-6 receptor (IL-6R), gastrocnemius, and myocardial IL-6R were increased following exercise in C57 mice only. Phosphorylated (p) signal transducer and activator of transcription 3 was increased in gastrocnemius and heart in C57 and IL-6−/− mice postexercise, whereas myocardial iNOS and cyclooxygenase-2 were unchanged in the exercised myocardium. Exercise protected C57 EX mice against I/R-induced arrhythmias and necrosis, whereas arrhythmia score and infarct outcomes were higher in C57 SED, IL-6−/− SED, and IL-6−/− EX mice compared with SH. C57 EX mice expressed increased p-44/42 MAPK (Thr202/Tyr204) and p-p38 MAPK (Thr180/Tyr182) compared with IL-6−/− EX mice, suggesting pathway involvement in exercise preconditioning. Findings indicate exercise exerts cardioprotection via IL-6 and strongly implicates protective signaling originating from the exercised skeletal muscle.

cardioprotection; myokine; myocardial infarction

CARDIOVASCULAR DISEASE remains a leading cause of morbidity in the United States and most predominately manifests as the myocardial infarction or ischemia-reperfusion (I/R) injury (57). Clinical outcomes subsequent to I/R accrue in a time-dependent fashion (6, 27, 44), beginning with ventricular arrhythmias and rapidly proceeding to irreversible cell death by apoptosis and necrosis (7). Autophagic processes also determine cellular fates during myocardial I/R injury (17, 20, 21). Clinical countermeasures to the cellular pathology that underpin I/R require novel understanding of robust and sustainable approaches to cardiac preconditioning via pharmacological and lifestyle countermeasures. Over the last 30 years ischemic preconditioning (IPC) (46) has been the primary investigative avenue to uncover viable countermeasures to I/R injury. Exercise has emerged in recent years as an alternative scientific model that exhibits mechanistic differences from IPC (15, 53), yet is just as effective as a cardioprotective stimulus.

From a phenomenological perspective, exercise preconditioning provides a more sustained window of protection than IPC (36) and protects the aged myocardium (52), whereas IPC does not (58). Understanding the endogenous mechanisms of exercise-induced cardioprotection has been the subject of intense inquiry in recent years (10, 15, 24, 25, 29, 37, 50, 55), yet remains incompletely understood. Several essential mediators to the exercised heart include endogenous antioxidants (16, 24, 25, 51) and ATP-sensitive potassium channels (5, 55, 56). Recent findings suggest that exercise-induced preconditioning is triggered by receptor-mediated events, including cardiac production of endogenous opioid compounds (11, 42). Given recent interest in acute exercise and skeletal muscle-derived “myokines,” there is a rationale to believe exercise preconditioning may occur through paracrine and endocrine-like processes originating in the exercised muscle, although this process remains untested (39, 47–49, 61). In the context of exercise, interleukin-6 (IL-6) is perhaps the most notable myokine with cardioprotective potential (30, 49, 61) and has been implicated as an essential trigger of IPC (9, 40, 59). Just as intriguing as IPC, and perhaps with more immediate clinical ramifications, remote preconditioning of the heart is an established physiological response to intermittent ischemia of limb skeletal muscle (8). Given that muscle-derived compounds are cardioprotective against myocardial infarction (4, 31), there is reason to suspect that a similar phenomenon may occur due to exercise.

Mechanistic studies of IL-6-induced cardioprotection have been carried out both in vivo (9) and in vitro (59), pharmacologically and via IPC. IPC-induced signaling through the Janus kinase-signal transducer and activator of transcription (JAK/STAT) pathway reduced necrosis following 30 min regional ischemia (9). The infarct-sparing effects were abrogated in IL-6−/− mice, demonstrating an essential role for IL-6 in IPC (9). Bolli et al. developed a sophisticated mouse model with a cardiac-specific, inducible STAT3 deletion and demonstrated the importance of STAT3 signaling in IPC (3). Smart et al. further demonstrated IL-6-induced cardioprotection against I/R injury in vitro. They observed a reduction in cellular damage, preservation of cell viability, and Ca2+ homeostasis following I/R (60). The protection afforded to the cardiomyocytes was nitric oxide (NO) and phosphatidylinositol

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H1423
3-kilobase (PI3K) dependent and was abolished by iNOS and PI3K inhibitors (59).

This collective understanding serves as the scientific rationale for the postulate that exercise-induced increases in circulating IL-6, largely attributed to increased synthesis and release from exercising skeletal muscle, are sufficient to activate protective pathways in the myocardium. Therefore, the purpose of this study was to investigate the acute postexercise period of IL-6 signaling in the heart and to study the role of IL-6 in exercise-induced cardioprotection against I/R injury using an IL-6 knockout mouse model. In that regard, it was hypothesized that exercise will increase IL-6 signaling in the heart and decrease I/R-induced apoptotic and necrotic tissue death in an IL-6-dependent fashion.

METHODS

Animals

Male mice (56 C57, C57BL/6J and 48 IL-6-/-/ from the same background strain, B6;129S2-IH6m1Kopf/J) were used to complete the two aims of this study. Auburn University Institutional Animal Care and Use Committee approval was granted before the start of the investigation and in accordance with National Institutes of Health guidelines for the care and use of laboratory animals. Animals were housed at the Auburn University Biological Research Facility on a 12:12-h reversed light-dark cycle with access to water and rodent chow ad libitum. In search of a homeostatic equilibrium, mice habituated to treadmill exercise for 10, 20, 30, and 40 min on consecutive days, followed by a rest day and then performed the 3-day treatment of exercise for 60 min/day at 18 m/min and 0% grade. Exercise was performed on a calibrated motorized rodent treadmill (Columbus Instruments, Columbus, OH), and sedentary mice, used as preexercise controls, spent a time-matched duration on the treadmill at 0 m/min. Under isoflurane anesthesia, tissue was collected from sedentary mice (PRE) and exercised mice immediately postexercise (POST) and 30 or 60 min postexercise. Blood serum, gastrocnemius, soleus, extensor digitorum longus (EDL), and hearts were snap-frozen for subsequent analysis. Mice in aim 1 (C57 n = 24 and IL-6-/-/ n = 16) followed an identical habituation and preconditioning exercise protocol and received an in vivo I/R injury 24 h following the final exercise session.

In Vivo I/R Injury

Twenty-four hours following the final exercise session, mice received surgically induced I/R injury or a time-matched sham operation. Mice were anesthetized using pentobarbital sodium (50 mg/kg), and a tracheotomy and left thoracotomy were performed. Mice were supported with a pressure-driven mechanical ventilator (Kent Scientific, Torrington, CT) and connected to limb lead electrodes integrated into a physiological data acquisition system (Biopac, Santa Barbara, CA) to record electrocardiogram (ECG) activity. The left anterior descending coronary artery was occluded with a sterile surgical suture passed through polyethylene tubing, creating a reversible ligature. Regional ischemia was administered for 30 min following by 120 min reperfusion. At the conclusion of I/R, the ligature was reestablished, and 4% Evan’s blue dye was injected via left ventricular cardiac puncture allowing for visualization of the area at risk (AAR). An additional group of C57 mice (n = 8) received a time-equivalent sham operation.

ECG data were collected and analyzed for ventricular arrhythmias using Biopac software and evaluated using the A Score method (43), a scoring system designed to categorize severity of I/R based on the incidence of premature ventricular contractions (PVCs), episodes and duration of ventricular fibrillation (VF), and tachycardia (VT) and accounting for mortality. To assess myocardial necrosis, hearts were excised, sectioned into 2-mm transverse cross sections, and incubated with 1% triphenol tetrazolium chloride (TTC) for 15 min at 37°C. To assess apoptosis and autophagy, the ischemic and perfused areas of the myocardium were separated and snap-frozen in liquid nitrogen for Western Blotting and PathScan analysis.
Table 1. **PCR Primers**

<table>
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<tr>
<th>Gene</th>
<th>Forward Primers (F)</th>
<th>Reverse Primers (R)</th>
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<td>TCCAAATTTCCGAGAAGAC</td>
</tr>
<tr>
<td>LIF (variant 1)</td>
<td>AGTAGCCGACTTGGGTCCTTCT</td>
<td>GGACAGTCGCGTGGTCAAA</td>
</tr>
<tr>
<td>Cfl3 (CT-1)</td>
<td>CCAATCTGTTCTTGGGTGAT</td>
<td>TCTTAAGGTTGAAAGGCTTG</td>
</tr>
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<td>IL-6R</td>
<td>CAGTGGCTGAGTCTCTTCTTCT</td>
<td>GAAATTTGCACTGCGTAAAC</td>
</tr>
<tr>
<td>Ptg2 (COX-2)</td>
<td>AGAGAAGAGTGGTGGCAAGAA</td>
<td>GCTGGTGTTCCTGAGTAAGA</td>
</tr>
<tr>
<td>NOS2 (iNOS)</td>
<td>CACCTTGGATCTACACCAAGT</td>
<td>AGCCATGTGACTGGGAGTC</td>
</tr>
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</table>

Primers were selected using Primer3Plus freeware and ordered from IDT Technologies.

**Serum IL-6 ELISA**

Serum IL-6 was quantified using a commercially available ELISA (KMC0062; Invitrogen) following the manufacturer’s protocol. Briefly, blood from aim 1 was collected via direct cardiac puncture and allowed to clot. Samples were centrifuged for 10 min at 10,000 g at 4°C and stored at −80°C until analysis. Serum samples were run in duplicate with the absorbance read at 450 nm and plotted against a standard curve of mouse IL-6 ($r^2 = 0.998$).

**PCR**

Approximately 20 mg of myocardial, gastrocnemius, soleus, and EDL tissue were used for RNA isolation using 500 μl RiboZol RNA Extraction Reagent (Ambion, Austin, TX) per the manufacturer’s instructions. RNA was precipitated with 500 μl isopropanol with 0.5 μl glycogen, ethanol washed, and reconstituted with diethylpyrocarbonate H2O. A 3% agarose gel was run to verify extracted RNA purity. Isolated RNA (1 μg) was converted to cDNA using a Verso cDNA kit (AB-1453/B; Thermoscientific) with a reverse transcription cycle of 30 min at 42°C. The resulting cDNA (50 ng/μl) was diluted to a final concentration of 5 ng/μl and stored at −20°C. Primer efficiency curves were run to select optimum cDNA concentrations and ensure single amplification products. PerfeCTa SYBR Green Super Mix (95054; Quanta BioSciences) was combined with forward and reverse primers (Table 1) and 25 ng cDNA. qPCR conditions were 95°C for 2 min, followed by 35 cycles of denaturing at 95°C for 30 s, reannealing at 62°C for 30 s, and extension at 72°C for 5 min. Relative mRNA expression was calculated using the ΔΔCt method for $n = 7–9$ observations/group.

**Western Blotting**

Serum, gastrocnemius, and heart (aim 1) samples were homogenized in lysis buffer with phosphatase (1:100; GiboSciences Phosphatase Arrest II) and protease (no. P2714, 1:10; Sigma) inhibitors to acquire a whole muscle homogenate. Cytosolic and nuclear fractions were obtained using a nuclear isolation kit (ThermoScientific, Waltham, MA) following the manufacturer instructions. Ischemic and perfused regions of hearts (aim 2) were homogenized in PathScan PhosphoSerine, Threonine, or Tyrosine Phosphoprotein kit (Cell Signaling) and normalized in lysis buffer with phosphatase (1:100; GiboSciences Phosphatase Arrest II) and protease (no. P2714, 1:10; Sigma) inhibitors. Samples were normalized for protein concentration, diluted with Laemmli sample buffer with 2-mercaptoethanol, and heated at 95°C for 5 min.

Proteins were separated on 6% iNOS, 10% IL-6 receptor (IL-6R), cyclooxygenase-2 (COX-2), phosphorylated (p)-STAT3, p-Akt, Atg3, Atg5, beclin 1, or 18% (LC3II/I) polyacrylamide gels and transferred to methanol-activated PVDF membranes. Membranes were exposed to primary antibodies [IL-6R (no. 374259; Santa Cruz), 1:1,000 in 5% nonfat dry milk (NFDM); p-STAT3 (no. 9145; Cell Signaling); STAT3 (no. 4904; Cell Signaling); p-Akt (no. 9271; Cell Signaling); Akt (no. 9272; Cell Signaling); iNOS (no. 13120; Cell Signaling); COX-2 (no. 12282; Cell Signaling); Atg3 (no. 3415; Cell Signaling); Atg5 (no. 12994; Cell Signaling); beclin 1 (no. 3495; Cell Signaling); LC3II/I (no. 12741; Cell Signaling); 1:1,000 in 5% BSA; and α-tubulin (no. 12G10; Developmental Studies Hybridoma Bank), 1:1,000 in 5% NFDM] overnight at 4°C. Membranes were incubated in mouse or rabbit targeted secondary antibodies (nos. 7076 and 7074, respectively, 1:2,000 in BSA; Cell Signaling) for 1 h at room temperature (RT), and blots were imaged with Luminata Forte HRP substrate (Millipore). Images were captured with a ChemiDoc-IT Imager (UV, Upland, CA) and analyzed using National Institutes of Health (NIH) ImageJ software.

**PathScan**

PathScan slides were assembled in gaskets and blocked for 15 min at RT. ISC and perfused homogenates, diluted to 1.0 mg/ml, were added to wells and incubated overnight at 4°C with rocking. Wells were incubated with a detection antibody followed by HRP-linked streptavidin with four 5-min washes separating each step. Slides were exposed with LumiGLO/Peroxide substrate, quantified using UVP software, and normalized to the total intensity of each sample.

**Statistical Analysis**

All values are presented as means ± SE. A 2 (genotype) × 4 (time) ANOVA was used to analyze effects of genotype and exercise on mRNA and protein expression following exercise. Arrhythmia scores were categorized using a nonparametric scoring method and were compared using a Kruskal-Wallis test for nonparametric data. Repeated-measures ANOVA was used to analyze group differences in AAR, infarct size (necrosis), and apoptosis with Tukey’s honest significant difference post hoc analysis to evaluate significant differences when appropriate. Significance was set a priori at $P = 0.05$.

**RESULTS**

**Aim 1**

**Serum exercise response.** Consistent with previous findings, serum IL-6 was increased ~4.5-fold 30 min postexercise (from 1.7 ± 0.4 to 7.5 ± 2.4 pg/l, Fig. 2). Exercise increased the soluble form of the IL-6 receptor (sIL-6R) in serum 4.5 ± 0.4-, 4.5 ± 0.8-, and 5.0 ± 0.6-fold at POST, 30 min, and 60 min, respectively, in C57 mice only (interaction effect, $P = 0.003$). Preexercise sIL-6R levels were similar between mouse strains, but significantly higher in C57 mice compared with IL-6−/− mice at all sample times postexercise (Fig. 3).
IL-6R expression was increased at POST and 30 and 60 min, but no differences were seen PRE. Open bars are C57 mice, and shaded bars are IL-6-/-. The nuclear fraction of gastrocnemius homogenate was analyzed for p-STAT3 (Tyr705). A significant increase in p-STAT3 was present at 30 min in C57 and IL-6-/− mice (time main effect, P = 0.003). Post hoc analysis revealed an increase in p-STAT3 at 60 min postexercise in C57 mice only. Interestingly, there was no effect for strain (P = 0.392), demonstrating that exercise induced p-STAT3 in C57 and IL-6-/− mice.

Examination of iNOS expression demonstrated a modest but significant increase in response to exercise (time main effect, P = 0.001) at all sample times postexercise in C57 and IL-6-/− mice. Follow up t-tests revealed a 1.5 ± 0.09-fold increase POST (P = 0.001), 1.6 ± 0.09-fold increase at 30 min (P < 0.001), and 1.4 ± 0.08-fold increase at 60 min (P = 0.005). Gastrocnemius COX-2 was significantly higher postexercise compared with 60 min (time main effect; P = 0.003). No strain differences were seen in the expression of iNOS (P = 0.838) or COX-2 (P = 0.587).

Real-time qPCR results for gastrocnemius are presented in Table 2. IL-6 gene expression in the gastrocnemius did not reach statistical significance but was numerically doubled at 30 min postexercise. Transcript expression for the alternative IL-6 family cytokines cardioprophchin-1 (CT-1) and leukemia inhibitory factor (LIF) was unaffected by exercise in C57 or IL-6-/−

**Gastrocnemius exercise response.** Western blotting and RT-PCR were used to examine the expression of proteins associated with IL-6 signaling in response to the acute exercise stimulus in gastrocnemius and the myocardium. Mixed gastrocnemius was assayed as an index of skeletal muscle IL-6 signaling (Fig. 4). Exercise elicited an increase in gastrocnemius IL-6R expression (strain main effect, P < 0.001 and time main effect, P = 0.042). IL-6R expression was increased 2.2 ± 0.5-fold POST, 2.4 ± 0.4-fold at 30 min, and 2.3 ± 0.3-fold at 60 min compared with preexercise in C57 mice, and was significantly higher than IL-6−/− animals at all sample times postexercise. The nuclear fraction of gastrocnemius homogenate was analyzed for p-STAT3 (Tyr705). A significant increase in p-STAT3 was present at 30 min in C57 and IL-6−/− mice (time main effect, P = 0.003). Post hoc analysis revealed an increase in p-STAT3 at 60 min postexercise in C57 mice only. Interestingly, there was no effect for strain (P = 0.392), demonstrating that exercise induced p-STAT3 in C57 and IL-6−/− mice.

**Serum IL-6R**. Serum was normalized for protein concentration via Bradford assay and analyzed via Western blotting for sIL-6R. Exercise increased sIL-6R at all time points in C57 mice only. C57 concentration via Bradford assay and analyzed via Western blotting for sIL-6R. Exercise increased sIL-6R at all time points in C57 mice only. C57
IL-6 mRNA expression was increased at 60 min postexercise in C57 and IL-6−/− mice (P < 0.001). A significant interaction was noted in iNOS expression (P = 0.004). Subsequent post hoc analysis revealed time and group effects were driven by significantly higher basal iNOS expression in IL-6−/− mice compared with all other groups, whereas no differences existed between any other sample times. COX-2 mRNA expression was higher postexercise in the IL-6−/− mice compared with PRE, 30 min, and 60 min, and was also increased compared with C57 POST.

**Exercised myocardium.** IL-6R protein expression was increased in the myocardium (interaction effect, P = 0.037). IL-6R expression was increased 2.6 ± 0.5-fold POST, 2.1 ± 0.4-fold at 30 min, and 2.6 ± 0.3-fold at 60 min in C57 mice only, and was increased compared with IL-6−/− mice at each time point postexercise. No difference in IL-6R was present between C57 and IL-6−/− mice PRE (Fig. 5A). p-Akt (Ser473) was measured in the myocardium as an index of IL-6-induced signaling through the PI3K/Akt pathway. Interestingly, in both groups, p-Akt levels were lower POST and returned to preexercise levels by 30 and 60 min postexercise (Fig. 5B). p-STAT3 (Tyr705) was increased in both groups at 30 min postexercise. Additionally, p-STAT3 in C57 mice was increased at 60 min and was significantly higher than IL-6−/− mice. IL-6−/− mice showed an earlier peak in p-STAT3 that increased immediately postexercise, but was no longer elevated by 60 min postexercise (Fig. 5C).

No differences were present for myocardial COX-2 in response to exercise. Cardiac levels of iNOS were decreased 60 min postexercise in both groups (time main effect; P = 0.039). No differences existed between C57 and IL-6−/− mice for either COX-2 or iNOS (Fig. 5, D and E, respectively).

Myocardial RT-PCR data are presented in Table 2. No increase in IL-6 mRNA expression was present postexercise. However, a postexercise increase in LIF mRNA existed in hearts harvested 60 min postexercise for both C57 and IL-6−/− mice. Interestingly, myocardial CT-1 mRNA was elevated in the IL-6−/− mice at all sample times (group main effect; P = 0.044). Exercise elicited an increase in myocardial IL-6R mRNA at 30 (P < 0.001) and 60 (P < 0.001 from PRE, P = 0.048 from POST) min in C57 and IL-6−/− mice. iNOS mRNA expression was reduced POST (P = 0.022), 30 min (P = 0.001), and 60 min (P = 0.002) postexercise. No changes in myocardial COX-2 mRNA expression were present. The mRNA expression of suppressor of cytokine signaling-3, the negative feedback inhibitor for IL-6 signaling, was unchanged by exercise.

**Aim 2**

**Mice anthropometrics.** Mice used for I/R investigation were separated into sedentary (C57 SED and IL-6−/− SED) or exercised (C57 EX and IL-6−/− EX) treatments, with a group of C57 mice used as a time-equivalent sham control (SH). Mice were weighed before I/R, and the hearts were blotted dry and weighed following cardiac excision; the data are presented in Table 3. IL-6−/− mice had a significantly higher body weight than C57 mice (30.3 ± 0.9 vs. 28.3 ± 0.5 g; P = 0.026), with no effect of exercise treatment (P = 0.396). IL-6−/− EX mice had a larger heart weight compared with sedentary mice, as well as C57 EX mice. Exercise increased the heart weight-to-body weight ratio in IL-6−/− mice (3.9 ± 0.2 to 4.6 ± 0.2), but not C57 mice (4.3 ± 0.1 to 4.6 ± 0.1).

**Arrhythmia scoring.** ECG data were analyzed under blinded conditions for PVCs, VT, and VF. Treatments were evaluated based on a composite arrhythmia scoring system for clinical and research settings (A Score) such that higher scores reflect more ventricular ectopy and account for mortality (43). C57 SED mice had a significantly higher ECG score than SH (P = 0.007). C57 EX mice were protected against arrhythmias, had significantly lower ECG scores than the C57 SED mice (P = 0.007), and were not statistically different from SH. IL-6−/− SED mice had a higher ECG score compared with SH mice (P = 0.005). The increase in arrhythmia score in IL-6−/− EX mice approached significance (P = 0.077), but was not statistically different from SH (Fig. 6).

**TTC staining for necrosis.** Evan’s blue dye was used to quantify the AAR, and TTC staining identified the percent infarct. Digital images were analyzed with ImageJ (NIH). No differences in the AAR were present between groups (%heart area: SH 34.6 ± 2.8, C57 SED 35.6 ± 2.8, IL-6−/− SED 28.6 ± 3.1, C57 EX 40.2 ± 4.4, and IL-6−/− EX 38.9 ± 4.2, P = 0.208; Fig. 7A). In response to I/R, TTC analyses revealed a significant increase in necrosis in C57 SED (22.2 ± 4.0%, P =

### Table 2. Aim 1 PCR results from gastrocnemius and myocardium

<table>
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<tr>
<th>Tissue</th>
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<th>POST</th>
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</table>

Values are means ± SE; n = 6 experiments. PRE, sedentary; POST, postexercise; 30, 30 min postexercise; 60, 60 min postexercise. Note increase in myocardial and gastrocnemius IL-6 receptor (IL-6R) mRNA in both strains. Myocardial CT-1 was higher in IL-6−/− mice. Myocardial iNOS and COX-2 were not increased following exercise. *Different from PRE. #Different from POST. †Different from IL-6−/−. doi:10.1152/ajpheart.00850.2014 • www.ajpheart.org
0.001) and IL-6\textsuperscript{-/-} SED (22.7 ± 4.4%, \textit{P} = 0.008) compared with SH (2.8 ± 0.7%). C57 EX mice (7.8 ± 1.8%) exhibited no increase in necrosis compared with SH (\textit{P} = 0.867), whereas IL-6\textsuperscript{-/-} EX (20.2 ± 4.9%) mice had a larger infarct compared with SH (\textit{P} = 0.02) and C57 EX (\textit{P} = 0.049) (Fig. 7B).

**Autophagy.** Western blotting for markers of autophagy (Atg3, Atg5, LC3II/I, and beclin 1) are presented in Fig. 8. Cardiac Atg3 levels were lower in the ischemic tissue of C57 and IL-6\textsuperscript{-/-} mice in both exercise and sedentary groups (\textit{P} < 0.001). Additionally, IL-6\textsuperscript{-/-} EX had increased Atg3 compared with IL-6\textsuperscript{-/-} SED. No significant differences existed for Atg5, beclin 1, or LC3II/I in response to exercise in C57 or IL-6\textsuperscript{-/-} mice.

**Cell stress and apoptosis PathScan.** The Cell Stress and Apoptosis PathScan array was used as a simultaneous measure of multiple targets in associated pathways (\textit{n} = 7–9/group). No differences were found between sham ischemic and sham perfused tissues for any assay target; thus, the total SH average was used to calculate a fold change value for each target. Cleaved caspase-3 (Asp\textsuperscript{175}, \textit{P} = 0.022) and caspase-7 (Asp\textsuperscript{198}, \textit{P} = 0.007) were increased in ischemic tissue of C57 and IL-6\textsuperscript{-/-} mice, with no difference between sedentary and exercised treatments. p-SAPK/JNK (Thr\textsuperscript{183}/Tyr\textsuperscript{185}) was increased in ischemic tissue of C57 and IL-6\textsuperscript{-/-} mice in sedentary and exercised groups (\textit{P} = 0.011) (Table 4).
protection was dependent on IL-6, and was abrogated in jury, we demonstrate for the first time that exercise-induced vents arrhythmias and myocardial necrosis following I/R in-

I/R injury. Although it is well established that exercise pre-

whether IL-6 is involved in exercise preconditioning against IL-6R protein expression is regulated in an IL-6-dependent 

tively. To our knowledge, this is the first study to demonstrate 

skeletal muscle IL-6R in the serum and gastrocnemius, respec-

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IL-6 

Fig. 6. Arrhythmia score. ECG were analyzed under blinded conditions. C57 sedentary and IL-6−/− SED mice had significantly higher arrhythmia scores. C57 exercised mice had a significantly lower score compared with C57 SED. A nonsignificant (NS) increase in arrhythmia score was seen in IL-6−/− EX. Open bars are C57 mice, and shaded bars are IL-6−/− mice. *Different from sham (SH). #Different from SED.

Cleaved PARP (Asp214) was reduced in C57 EX mice compared with IL-6−/− EX mice, but showed no differences between ischemic or perfused tissue, or between sedentary and exercised treatments in each strain. Cardiac p-p44/42 MAPK (Thr202/Tyr204) was lower in ischemic tissue from both strains and both sedentary and exercised mice (P = 0.027). C57 EX mice exhibited increased p-p44/42 MAPK compared with IL-6−/− EX mice, whereas no strain differences were present in sedentary mice. Similarly, a significant increase in p-p38 MAPK (Thr180/Tyr182) occurred in C57 EX compared with C57 SED and IL-6−/− EX (Fig. 9). No significant differences were present for Akt (Ser473), Bad (Ser136), HSP27 (Ser82), Chk1 (Ser82), Chk2 (Thr68), eIF2α (Ser51), TAK1 (Ser412), or survivin (total and Ser 32/36), Smad2 (Ser465/467), p53 (Ser 15), IκBα (total and Ser32/36), Chk1 (Ser145), Chk2 (Thr68), eIF2α (Ser51), TAK1 (Ser112), or survivin (total) (data not shown).

DISCUSSION

The current study was undertaken to 1) characterize exercise-induced IL-6 signaling in the heart and 2) to investigate whether IL-6 is involved in exercise preconditioning against I/R injury. Although it is well established that exercise prevents arrhythmias and myocardial necrosis following I/R injury, we demonstrate for the first time that exercise-induced protection was dependent on IL-6, and was abrogated in IL-6−/− mice. Furthermore, the current study findings reinforce that 1) exercise increases circulating levels of IL-6 protein and 2) exercise elicits an increase in soluble and skeletal muscle IL-6R in the serum and gastrocnemius, respectively. To our knowledge, this is the first study to demonstrate that exercise increases myocardial IL-6R protein, and that IL-6R protein expression is regulated in an IL-6-dependent fashion.

Cardioprotection

The current study is the first study to implicate exercise-induced IL-6-dependent protection against arrhythmias during I/R. This finding is in line with previous in vitro studies that strongly implicate that IL-6 has positive effects on contractility (41) and calcium handling in culture (59). Perhaps most interestingly, a large cohort genomewide association study (Cohorts for Heart and Aging Research in Genomic Epidemiology, or “CHARGE”) found a single nucleotide polymorphism in the IL-6R gene that was associated with atrial fibrillation (38). Presently, I/R-induced arrhythmias were increased in sedentary mice, and exercise conferred cardioprotection in C57 mice. A nonsignificant increase (P = 0.077) in arrhythmias in IL-6−/− EX mice makes it tempting to speculate that exercise-induced protection is IL-6 dependent, but that is not statistically supported in the current study. Although more mechanistic studies are needed, these early findings agree with others and suggest that IL-6 and IL-6R may impact arrhythmia prevention and/or protection.

In the current study we confirm that exercise mitigates I/R-induced myocardial infarction. Most importantly, however, we report for the first time that exercise-induced protection against tissue necrosis is dependent on IL-6. Although many
Fig. 8. Autophagy Western blotting. Ischemic (I) and perfused (P) myocardial samples were dissected and analyzed separately for autophagic biomarkers. There was a decrease in Atg3 in ischemic tissues, with an increase in IL-6−/− mice compared with SED (A). No changes were seen in Atg5 (B), beclin 1 (C), or LC3 (D). Open bars are C57 mice, closed bars are IL-6−/− mice, unhatched bars are perfused tissue, and hatched bars are ischemic tissue. *Different from SED. $Different from corresponding perfused tissues.

PathScan data

<table>
<thead>
<tr>
<th>PathScan Target</th>
<th>C57 Sedentary</th>
<th>IL-6−/− Sedentary</th>
<th>C57 Exercised</th>
<th>IL-6−/− Exercised</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P</td>
<td>I</td>
<td>P</td>
<td>I</td>
</tr>
<tr>
<td>JNK (Thr183/Tyr185)</td>
<td>1.48 ± 0.06</td>
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<td>1.51 ± 0.04</td>
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<td>Caspase3 (Asp75)</td>
<td>0.50 ± 0.05</td>
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<td>0.49 ± 0.08</td>
<td>0.53 ± 0.04*</td>
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<td>Caspase7 (Asp395)</td>
<td>1.28 ± 0.07</td>
<td>1.36 ± 0.09*</td>
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<td>Tubulin</td>
<td>1.14 ± 0.10</td>
<td>1.11 ± 0.07</td>
<td>1.21 ± 0.07</td>
<td>1.10 ± 0.09*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 7–9 experiments. P, perfused; I, ischemic. *Different from perfused.
and p38 was increased in ischemic and perfused tissue of C57 EX mice only, whereas this response was abolished in IL-6/or mice. That the MAPK response occurred in both ischemic and perfused tissues suggests that it was most likely due to preconditioning exercise and not I/R. Although the duration of ischemia was sufficient to induce myocardial injury, a more severe I/R injury or longer recovery period may have yielded more discrepant apoptosis-related outcomes. In support, previous studies by our laboratory using longer-duration I/R indicate exercise confers protection against apoptosis (16, 52, 54, 55).

**IL-6 Regulation and Signaling**

Exercise-induced increases in skeletal muscle IL-6 mRNA expression and circulating IL-6 protein are well documented (39, 47–49, 61). We observed the anticipated increase in serum IL-6, but did not detect statistically significant increases in IL-6 mRNA expression in either gastrocnemius or heart. Although we were unable to specifically account for the origin of the circulating IL-6, several key observations by others support a muscle-derived origin following exercise or contraction. Importantly, resting skeletal muscle contains significant quantities of IL-6 protein sequestered in storage vesicles that are released in response to stimulated contractions (34), which potentially explains the increased circulating IL-6 without increased mRNA. Furthermore, others found fiber type-specific increases (28), or no increase (1), in IL-6 mRNA in response to exercise, which could explain the present lack of sensitivity seen in mixed gastrocnemius. A subset of soleus and EDL was used to compare IL-6 mRNA production with gastrocnemius and heart. The heart showed the lowest expression of IL-6 followed by gastrocnemius, soleus, and EDL [2.3 ± 0.4 (not significant), 29.2 ± 4.8 (P < 0.001)–, and 61.5 ± 8.7 (P < 0.001)-fold higher, respectively; data not shown]. Finally, while circulating IL-6 is derived from other cell types, most notably immune cells, the transient IL-6 increase following exercise is not immune cell driven (45, 64). These data underpin our tentative conclusion that exercised skeletal muscle, rather than the myocardium, is the primary source of endogenous IL-6 release, although this assertion is not completely resolved in the current data and could be clarified using tissue-specific knockout models in the future.

Exercise-induced activation of IL-6 signaling pathways (JAK/STAT, Akt, and MAPKs) has been observed previously in response to exercise in skeletal muscle (63), but not in the heart. Surprisingly, we observed a rapid and transient induction of p-STAT3 in the gastrocnemius and heart of C57 and IL-6/or mice that returned to baseline values 60 min postexercise in IL-6/or mice but remained elevated in C57 mice. A possible explanation for these findings is that alternative IL-6 family cytokines (i.e., CT-1 or LIF) compensate for IL-6 loss. In support of that hypothesis, CT-1 was higher in IL-6/or mice compared with C57 or other IL-6 family cytokines.

Although PI3K/Akt/mammalian target of rapamycin signaling is associated with IL-6 and may be essential to some nonexercise models of IL-6-induced cytoprotection (59), we observed a decrease in cardiac p-Akt (Ser473) immediately postexercise in both C57 and IL-6/or mice. This response likely reflects a nonpathological bioenergetic exercise stimulus and agrees with the established role of 5’-adenosine monophosphate kinase (AMPK)-Akt balance as a molecular metabolic switch (2, 26). In a subset of exercised C57 mice, p-AMPKα (Thr172) was increased in the gastrocnemius and heart immediately postexercise (data not shown).

In conclusion, the current findings support an important role for IL-6 in exercise-induced cardioprotection against I/R injury. Furthermore, the current study sheds light on the regulation of the IL-6R in response to exercise and further suggests that upregulation of IL-6R is a necessary feature of the cardioprotected phenotype (12, 62). Subsequent investigations of the IL-6/exercise cardioprotection paradigm are needed in tissue-specific loss-of-function models (i.e., skeletal muscle-specific IL-6/or or cardiomyocyte-specific IL-6R/or or gp-130/or models) to expand upon these novel findings.
AUTHOR CONTRIBUTIONS

No conflicts of interest, financial or otherwise, are declared by the authors.

REFERENCES


