Interleukin-6 mediates exercise preconditioning against myocardial ischemia reperfusion injury

Graham Ripley McGinnis,1 Christopher Ballmann,1 Bridget Peters,1 Gayani Nanayakkara,2 Michael Roberts,3 Rajesh Amin,2 and John C. Quindry1

1Cardioprotection Laboratory, Auburn University School of Kinesiology, Auburn, Alabama; 2Department of Drug Discovery and Development, Auburn University Harrison School of Pharmacy, Auburn, Alabama; and 3Molecular and Applied Sciences Laboratory, Auburn University School of Kinesiology, Auburn, Alabama

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McGinnis GR, Ballmann C, Peters B, Nanayakkara G, Roberts M, Amin R, Quindry JC. 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-p44/42 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 phosphatidlyinositol
3-kinase (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-Ile6−/−Kopf/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 searching for a time-matched sham operation (SH).

**Aim 1**

**Exercise IL-6 Signaling**

(n=64)

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**Aim 2**

**Ischemia/Reperfusion**

(n=40)

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<td>C57 EX (n=8)</td>
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<td>IL-6−/− EX (n=8)</td>
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Fig. 1. Study design. **Aim 1**, C57 and interleukin-6 (IL-6−/−) mice were divided into sedentary (SED) and exercised (EX) groups and were killed PRE, POST, and 30 and 60 min postexercise by cardiac excision. **Aim 2**, exercised and sedentary C57 and IL-6−/− mice received surgically induced ischemia-reperfusion (I/R) injury with 30 min I and 120 min R. A group of C57 mice was used for a time-matched sham operation (SH). Open boxes are C57 mice, and shaded boxes are IL-6−/− mice.

**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 followed by 120 min reperfusion. At the conclusion of I/R, the ligature was reestablished, and 4% Evans’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

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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 (Amresco NS800) 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 optimal 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 30 s, reannealing at 62°C for 30 s, and extension at 72°C for 5 min. Relative mRNA expression was calculated using the $\Delta\Delta C_t$ 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; GIBiosciences 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 Sandwich ELISA Lysis buffer containing protease and phosphatase 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, Akt3, Akt5, beclin 1, or 18% (LC3II/I) polyacrylamide gels and transferred to nitrocellulose 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. 9271; Cell Signaling); Akt (no. 9272; Cell Signaling); iNOS (no. 13120; Cell Signaling); COX-2 (no. 12282; Cell Signaling); Akt3 (no. 3415; Cell Signaling); Akt5 (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 Luminanorte HRP substrate (Millipore). Images were captured with a ChemiDoc-IT Imager (UVP, 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. Ischemic 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 analyses to evaluate significant differences when appropriate. Significance was set a priori at $P \leq 0.05$.

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).
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 \( 2.2 \pm 0.5 \)-fold POST, \( 2.4 \pm 0.4 \)-fold at 30 min, and \( 2.3 \pm 0.3 \)-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.

Exercise-induced signaling in gastrocnemius. Western blotting was conducted on gastrocnemius samples. Whole muscle homogenates were used for IL-6R, nuclear fractions were used for phospho (p)-signal transducer and activator of transcription 3 (STAT3) and total STAT3 (presented as phospho-to-total protein ratio), and cytosolic fractions were used for iNOS and cyclooxygenase-2 (COX-2). Exercise elicited an increase in gastrocnemius IL-6R in C57 mice only (A), whereas a postexercise increase in the p-STAT3-to-STAT3 ratio was seen in both groups at 30 min and in C57 mice at 60 min (B). COX-2 was significantly higher POST compared with 60 min (C), and iNOS was increased at POST and 30 and 60 min (D). No differences were seen between C57 and IL-6/**−**/*−** mice in COX-2 and iNOS. Open bars are C57 mice and shaded bars are IL-6/**−**/*−** mice. *Different from PRE. †Different from POST. ‡Different from IL-6/**−**/*−**.
IL-6

Subsequent post hoc analysis revealed time and group effects. Cardiac levels of iNOS were decreased 60%

Increased immediately postexercise, but was no longer ele-

mRNA expression was reduced POST (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 also 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 treatment 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 =

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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-6R mRNA in both strains. Myocardial CT-1 was higher in IL-6/– mice. Myocardial iNOS and 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 preex-

crease levels by 10.2±0.3 on October 1, 2017 http://ajpheart.physiology.org/ Downloaded from
Fig. 5. Exercise-induced signaling in the myocardium. Western blotting was conducted on myocardial samples. Whole muscle homogenates were used for IL-6R, nuclear fractions were used for p-STAT3 and total STAT3 (presented as phospho-to-total protein ratio), and cytosolic fractions were used for p-Akt and total Akt (presented as phospho-to-total protein ratio), iNOS, and COX-2. Exercise increased IL-6R expression in C57 animals only (A). The p-Akt-to-Akt ratio was decreased postexercise and returned to baseline at 30 and 60 min (B). Exercise increased the p-STAT3-to-STAT3 ratio in C57 and IL-6/−/− mice (C). COX-2 was unchanged (D), whereas iNOS was suppressed (E). Open bars are C57 mice, and shaded bars are IL-6/−/− mice. *Different from PRE. #Different from POST. † Different from IL-6/−/−.

0.001) and IL-6/−/− SED (22.7 ± 4.4%, 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 (𝑃 = 0.867), whereas IL-6/−/− EX (20.2 ± 4.9%) mice had a larger infarct compared with SH (𝑃 = 0.02) and C57 EX (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/−/− mice in both exercise and sedentary groups (𝑃 < 0.001). Additionally, IL-6/−/− EX had increased Atg3 compared with IL-6/−/− SED. No significant differences existed for Atg5, beclin 1, or LC3II/I in response to exercise in C57 or IL-6/−/− 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 (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 (Asp175, 𝑃 = 0.022) and caspase-7 (Asp198, 𝑃 = 0.007) were increased in ischemic tissue of C57 and IL-6/−/− mice, with no difference between sedentary and exercised treatments. p-SAPK/JNK (Thr183/Tyr185) was increased in ischemic tissue of C57 and IL-6/−/− mice in sedentary and exercised groups (𝑃 = 0.011) (Table 4).

<table>
<thead>
<tr>
<th>Aim 2 mouse anthropometrics</th>
<th>C57</th>
<th>IL-6/−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sedentary</td>
<td>Exercised</td>
</tr>
<tr>
<td>Body wt, g</td>
<td>28.6 ± 0.7§</td>
<td>27.6 ± 0.6‡</td>
</tr>
<tr>
<td>Heart wt, mg</td>
<td>122.1 ± 2.6</td>
<td>126.0 ± 2.9*‡</td>
</tr>
<tr>
<td>Heart weight/body weight</td>
<td>4.3 ± 0.1</td>
<td>4.6 ± 0.1</td>
</tr>
</tbody>
</table>

Values are means ± SE. IL-6/−/− mice had slightly higher body wt, but no differences exist between C57 and IL-6/−/− heart-to-body wt ratio in either sedentary or exercised mice *Different from PRE. † Different from IL-6/−/−.
IL-6 protection was dependent on IL-6, and was abrogated in I/R injury. We demonstrate for the first time that exercise-induced protection against 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 mechanisms have been proposed to explain the cardioprotective effects of exercise, our work provides a mechanistic understanding of the role of IL-6 in this process.

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 (Ser345), Chk2 (Thr68), eIF2α (Ser51), and both sedentary and exercised mice (P = 0.027). C57 EX had a significantly lower score compared with C57 SED and IL-6−/− SED mice. *Different from SH. #Different from SED.

**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-induce protection against tissue necrosis is dependent on IL-6. Although many...
differences exist between exercise preconditioning and IPC-induced cardioprotection, IL-6 appears to be a common trigger. In support, Dawn et al. showed the infarct-sparing effects of IPC were mediated by IL-6 (9), and Gwechenberger et al. found increased IL-6 levels in the viable border zone surrounding infarcted tissue (23). Importantly, when IL-6–/– mice are exposed to IPC, STAT3 signaling and cardioprotection are eliminated. We currently demonstrate that STAT3 translocation in skeletal muscle and heart was not eliminated in exercised IP mice. Hence, the increase in soluble and myocardial IL-6R in C57 mice, which also showed increased serum IL-6 following exercise, was insufficient to elicit the same protection when administered post-I/R. However, other apoptotic markers, including caspase 3, caspase 7, and JNK, were increased in the ischemic tissues which also showed increased serum IL-6 following exercise, whereas IL-6 or the receptor alone was insufficient to elicit the same protection when administered individually (40). Furthermore, IL-6 could not induce STAT3 phosphorylation without the addition of the IL-6R in vitro (12). Hence, the increase in soluble and myocardial IL-6R in C57 mice, which also showed increased serum IL-6 following exercise, was insufficient to elicit the same protection when administered post-I/R. However, other apoptotic markers, including caspase 3, caspase 7, and JNK, were increased in the ischemic tissues independent of IL-6 or exercise, which makes these findings difficult to interpret (Table 4).

Independent of STAT3, IL-6 can induce phosphorylation of p44/42 MAPK (12). We found that phosphorylation of p44/42

Table 4. 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>
<td>1.66 ± 0.10*</td>
<td>1.51 ± 0.04</td>
<td>1.55 ± 0.09*</td>
</tr>
<tr>
<td>Caspase3 (Asp175)</td>
<td>0.50 ± 0.05</td>
<td>0.54 ± 0.05*</td>
<td>0.49 ± 0.08</td>
<td>0.53 ± 0.04*</td>
</tr>
<tr>
<td>Caspase7 (Asp199)</td>
<td>1.28 ± 0.07</td>
<td>1.36 ± 0.09*</td>
<td>1.24 ± 0.08</td>
<td>1.47 ± 0.05*</td>
</tr>
<tr>
<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-/- 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-/- mice that returned to baseline values 60 min postexercise in IL-6-/- 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. For instance, interstitial cell derived from IL-6-/- mice could be use for STAT3 signaling via increased basal CT-1, 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-/- 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 cardiomyocyte-specific IL-6R-/- or gp-130-/- models) to expand upon these novel findings.
INTERLEUKIN-6 AND EXERCISE PRECONDITIONING

GRANTS

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


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