Ischemia reperfusion injury, K\textsubscript{ATP} channels, and exercise-induced cardioprotection against apoptosis

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Submitted 29 July 2011; accepted in final form 28 May 2012

Quindry JC, Miller L, McGinnis G, Kliszczewicz B, Irwin JM, Landram M, Urbiztondo Z, Nanayakkara G, Amin R. Ischemia reperfusion injury, K\textsubscript{ATP} channels, and exercise-induced cardioprotection against apoptosis. J Appl Physiol 113: 498–506, 2012. First published May 31, 2012; doi:10.1152/japplphysiol.00957.2011.—Exercise is a potent stimulus against cardiac ischemia reperfusion (IR) injury, although the protective mechanisms are not completely understood. The study purpose was to examine whether the mitochondrial or sarcolemmal ATP-sensitive potassium channel (mito K\textsubscript{ATP} or sarc K\textsubscript{ATP}, respectively) mediates exercise-induced cardioprotection against post-IR cell death and apoptosis. Eighty-six, 4-mo-old male Sprague Dawley rats were randomly assigned to treadmill exercise (Ex; 30 m/min, 3 days, 60 min, ~70 maximal oxygen uptake) and sedentary (Sed) treatments. Rats were exposed to regional cardiac ischemia (50 min) and reperfusion (120 min) or Sham (170 min; no ligation) surgeries. Rats received placebo (saline), 5-hydroxydecanoate (5HD; 10 mg/kg/ip), or HMR1098 (10 mg/kg/ip) to inhibit mito K\textsubscript{ATP} or sarc K\textsubscript{ATP} channel. Comprehensive outcome assessments included post-IR ECG arrhythmias, cardiac tissue necrosis, redox perturbations, and autophagy biomarkers. No arrhythmia differences existed between exercised and sedentary hearts following extended-duration IR (P < 0.05). The sarc K\textsubscript{ATP} channel was confirmed essential (P = 0.002) for prevention of anectrotic tissue death with exercise (percent infarct, Sed = 42%; Ex = 20%; Ex5HD = 16%; ExHMR = 42%), although neither the mito K\textsubscript{ATP} (P = 0.177) nor sarc K\textsubscript{ATP} (P = 0.274) channel provided post-IR protection against apoptosis (terminal deoxynucleotidyl transferase deoxy UTP-mediated nick-end labeling-positive nuclei/mm\textsuperscript{2}; Sham = 1.8 ± 0.5; Sed = 19.4 ± 6.7; Ex = 7.5 ± 4.6; Ex5HD = 14.0 ± 3.9; ExHMR = 11.1 ± 1.8). Exercise preconditioning also appears to preserve basal autophagy levels, as assessed by Beclin 1 (P ≤ 0.001), microtubule-associated protein-1 light chain 3B ratios (P = 0.020), and P62 (P ≤ 0.001), in the hours immediately following IR. Further research is needed to better understand these findings and corresponding redox changes in exercised hearts.

autophagy; heart disease; infarction; myocardial; preconditioning

ISCHEMIC HEART DISEASE is a leading cause of morbidity in industrialized countries (30). Cardiac ischemia reperfusion (IR) injury manifests as an evolving series of physiologic events, where injury severity is proportional to increased ischemic duration. Ongoing research is directed at identifying the endogenous mechanisms of cardioprotection against IR injury. Myriad mechanisms provide cardioprotection through mitigation of oxidative stress and energy-supply mismatch experienced during IR (34). In particular, preservation of cellular redox balance during IR attenuates ECG abnormalities in the first moments of an ischemic event in addition to prevention of tissue death during extended insults (34). Related to both myocardial redox balance and energy availability, the mitochondrial and sarcolemmal ATP-sensitive potassium channels (mito K\textsubscript{ATP} and sarc K\textsubscript{ATP}, respectively) have received significant attention as potent, protective mediators against IR injury (8, 10, 12, 23-25, 38); however, sustainable activation of these channels has been elusive (3). K\textsubscript{ATP} channels are comprised of an inwardly rectifying transmembrane K\textsuperscript{+} ion channel and a sulfonflyreula regulatory unit positioned within the cytosol. The resulting octomeric complex is found on the inner mitochondrial and sarcolemmal membranes of cardiac muscle (44). A variety of cardiac-preconditioning strategies, including endurance aerobic exercise, have been used as countermeasures against IR injury. From a clinical perspective, exercise is an advantageous model for understanding cardioprotection against IR injury, because the stimulus is sustainable and pragmatic [reviewed in (9, 34, 36)].

From an experimental paradigm, cardiac preconditioning by short-term (days) exercise is a potent cardioprotective stimulus against ischemic injury (15). Exercise-induced cardioprotection against IR insults is multifaceted, although the mediators are only partially understood. The various mechanisms of exercise-induced cardioprotection appear specific to individuals, to the form of ischemic injury. For example, upregulation of the endogenous antioxidant enzyme manganese SOD (Mn-SOD) within ventricular tissue is essential for preventing IR-induced arrhythmias (21) and tissue death (18) but not losses in contractile dysfunction (29). A series of studies by our lab and others has examined the role of the K\textsubscript{ATP} channels in IR-injury resistance (7, 12, 23, 24, 38). We recently found that the mito K\textsubscript{ATP} channel, but not the sarc K\textsubscript{ATP} channel, mediated protection against IR-induced arrhythmias during a relatively short-duration IR event in vivo (38). In contrast, the sarc K\textsubscript{ATP} channel mediates protection against necrotic tissue death following regional IR in isolated hearts (7). Currently unknown is whether the mito K\textsubscript{ATP} or sarc K\textsubscript{ATP} channel is responsible for the antiapoptotic protection observed in exercised hearts post-IR (18, 35, 37). The purpose of this investigation was to examine whether the mito K\textsubscript{ATP} or sarc K\textsubscript{ATP} channel mediates exercise-induced cardioprotection against IR-induced apoptosis and tissue death. Experimental design considerations for the current investigation were used to provide new insight into the role of redox balance and autophagy in exercise-induced cardioprotection.

METHODS

Animals and experimental design. The experimental protocol was approved by the Appalachian State University Animal Care and Use
Committee and followed guidelines established by the American Physiological Society for the use of animals in research. Adult male Sprague-Dawley rats (~4 mo old) were randomly assigned to cardioprotective exercise or sedentary treatments. During the experimental period, all animals were housed on a 12-h light:12-h dark cycle and provided rat chow (AIN93) and water ad libitum.

**Exercise and sedentary group study treatments.** Eighty-six rats were randomly assigned to cardioprotective exercise (Ex; n = 54) or sedentary (Sed; n = 32) groups. Based on key dependent outcome measures, hearts from 43 animals were designated for post-IR biochemical assessment or post-IR histological assessment. Cardioprotective exercise is described subsequently. Sedentary animals remained in their vivarium cages without formal exercise. To normalize for handling stress, sedentary animals were placed on nonmoving treadmills for a time duration equal to exercised treatments. Exercised rats were further randomized into one of three treatments: saline (Sal; n = 18), 5-hydroxydecanoate (5HD; 10 mg/kg), a selective pharmacologic inhibitor of the mito KATP channel (“Ex5HD”), or placebo (“Ex”; n = 16). Studies were performed for handling stress, sedentary animals were placed on nonmoving treadmills for a time duration equal to exercised treatments.

**Cardioprotective exercise protocol.** Rats assigned to the exercise treatment received, over 10 days, an exercise regimen previously shown to elicit a cardioprotected phenotype against IR injury (18, 21, 37). The exercise regimen began with a habituation period of 5 consecutive days of treadmill exercise. Treadmill habituation involved a gradual increase in running time beginning on day 1 with 10 min of exercise. Successive days introduced time increases of 10 min/day concluding with 50 min of total exercise on the 5th and final day of treadmill habituation. At the conclusion of the treadmill habituation period, animals received 2 days of rest, followed by 3 consecutive days of 60-min exercise bouts. Treadmill speed and grade were fixed at 30 m/min and 0% grade for both treadmill habituation and exercise training. Sedentary (Sed) treatments.

**In vivo IR protocol.** Twenty-four hours following the exercise protocol, rats were exposed to a nonsurvival IR protocol involving coronary artery ligation in vivo, as described previously (18, 21, 37). Anesthetized rats (65 mg/kg Na\(^+\) pentobarbital ip) mechanically ventilated (Kent Scientific, Torrington, CT) with room air. A heated water blanket was used to maintain animal core temperatures at 37°C. The jugular vein was catheterized for supplemental pentobarbital (10 mg/kg, a selective pharmacologic inhibitor of the mito KATP channel (“Ex5HD”), or placebo (“Ex”). HMR1098 (10 mg/kg), a selective pharmacologic inhibitor of the sarc KATP channel (“ExHMR”). HMR1098 was a generous gift of Dr. Heinz Golgelein (Adventis Pharma, Gerrards). Hearts, as directed by previous research, isovolumetric placebo and pharmacologic doses were administered via intraperitoneal injection, 45 min prior to in vivo IR experimentation. Injection time provides a 15- to 30-min time buffer for known effects on channel activity (20, 38). Rats assigned to the sedentary treatment were randomized into “Sham” (no ischemia) or “Sed” treatments.

**Arrhythmia scoring.** Electrocardiographic tracings were analyzed for ventricular arrhythmias to assess the magnitude of ventricular ectopy as described previously (21, 38). Blinded digital ECG files were read by two trained reviewers who coded for preventricular contractions (PVCs), ventricular tachycardia, and ventricular fibrillation. An established arrhythmia scoring system was applied to ventricular arrhythmia outcomes in accordance with Lambeth Conventions guidelines previously used to assess clinical ECG responses to an ischemic insult (13, 14, 43).

**Analysis of infarct and apoptosis.** Hearts from subsets of animals receiving IR and Sham protocols were used to assess tissue death from necrosis and apoptosis. Necrotic tissue death was evaluated using a triphenyl tetrazolium chloride (TTC) technique. Following Evan’s blue infusion and excision, hearts were sliced into thin cross-sections (~1.5 mm) and incubated in 1% TTC-saline solution for 10 min at 37°C. Heart cross-sections were digitally imaged to obtain total, at risk, and infarcted cross-sectional areas using a Kodak image analysis device. All images were viewed under blinded conditions by two study personnel, and averaged values were obtained for the percent of the area at risk (%AAR: AAR/total area) and the percent infarction (TTC-positive area/AAR). Final values represent averages of four to five ventricular cross-sections taken from apex to base for each heart.

**Histological sections coincubated with the TUNEL enzyme label** were embedded in Neg-50 Frozen Section Medium and stored at ~80°C for subsequent analysis. Tissue was cryosectioned (8 μm) in a Microm cryotome and assayed for TUNEL using a histochemical fluorescent detection kit (Roche Applied Scientific, Indianapolis, IN). Sections were fixed with 10% formalin, washed, and permeabilized with Triton X-100 in a 1% sodium citrate solution. Samples were blocked with 3% BSA and 20% goat serum. Histological sections were normalized to tissue cross-sectional area. Histological images without en face tissue coverage were normalized to the area present.

**Western blot analysis for markers of apoptosis and autophagy.** Myocardial protein levels of apoptosis and autophagy biomarkers were performed via Western blot. Briefly. ~20 μg myocardial protein/sample was separated using standard SDS-PAGE techniques on a polyacrylamide gel. Proteins were transferred to polyvinylidene difluoride membranes and exposed to primary antibodies for B cell lymphoma 2 (Bcl2)–associated X protein (Bax)/Bcl2, Belcin 1, microtubule-associated protein-1 light-chain 3B (LC3BII/LC3BI, and autophagy-related (Atg)5, Atg7, and Atg12 (Cell Signaling Technology, Danvers, MA) raised in rabbit. Antibodies directed against the autophagy protein P62 were also used (Sigma-Aldrich). An anti-rabbit IgG-horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology) was used for chemiluminescence detection using a Kodak digital imaging system (Rochester, NY). All blots were re-probed for β-actin (Cell Signaling Technology) and individual samples normalized to these values. Ischemic and perfused ventricular samples from each group were run on multiple gels with exercised and sedentary samples expressed as a percentage of the sedentary hearts.

**Analysis of antioxidant enzyme activity and oxidative stress.** Myocardial antioxidant enzyme activities were examined in perfused and nonperfused left ventricular tissues. Muscle was homogenized on glass on ice-cold 100 mM phosphate buffer (1:20 wt:vol, pH 7.4), centrifuged at 400 g for 10 min at 4°C, and supernatants assayed for total protein content (6). The activities of SOD and its respective CuZn (CuZnSOD) and MnSOD isoforms (31), glutathione peroxidase (GPX) (17), and catalase (CAT) (1) were examined. All analyses were performed using standard biochemical techniques.
performed at 25°C on the same day to avoid interassay variation. The assay coefficients of variation for SOD, GPx, and CAT assays ranged between 2% and 5%. Myocardial protein carbonyl formation was assessed using a commercially available ELISA (Northwest Life Sciences, Vancouver, WA).

Data analysis. One-way ANOVA was used to evaluate perfused and nonperfused tissue areas, arrhythmia scoring data and ventricular ectopy data, necrotic tissue death, and TUNEL outcomes. Two-way repeated measures ANOVA was used to evaluate perfused and non-perfused variables. When appropriately indicated by the presence of significant main effects and interaction effects, group differences were determined via Tukey post hoc analysis. Significance was established a priori at $P < 0.05$.

RESULTS

Animal characteristics. Eighty-two of the 86 animals used in this investigation were included in the final data analysis. Animal attrition was due to complications experienced during the IR protocol ($n_{\text{Sed}} = 1$; $n_{\text{Ex}} = 1$; $n_{\text{Ex5HD}} = 2$; $n_{\text{ExHMR}} = 9$). Of these animals, 40 of 43 hearts designated for biochemical assay ($n_{\text{Sham}} = 6$; $n_{\text{Sed}} = 8$; $n_{\text{Ex}} = 9$; $n_{\text{Ex5HD}} = 9$; $n_{\text{ExHMR}} = 9$) were used, whereas 42 of 43 hearts designated for histological assessment ($n_{\text{Sham}} = 5$; $n_{\text{Sed}} = 9$; $n_{\text{Ex}} = 9$; $n_{\text{Ex5HD}} = 10$; $n_{\text{ExHMR}} = 9$) were used. Animal treatment numbers and body weights are presented in Table 1. Statistical analysis revealed that body weights were similar for all treatments. Analysis of the %AAR for infarction (Fig. 1A) demonstrates all IR treatments experienced statistically similar involvement of ischemic and perfused tissue ratios. The ratio of perfused to “nonperfused” was similar between hearts exposed to Sham surgery treatment and all IR heart treatments ($P = 0.244$).

Electrocardiographic activity during IR and Sham treatments. Ventricular arrhythmias were observed within minutes of surgically induced ischemia in all animals, with the majority of ectopic events occurring during ischemia. A few Sham animals experienced a limited number of PVCs, presumably in response to passing the surgical ligature through excitable ventricular tissue during the surgical procedure. No Sham animals experienced ventricular tachycardia or ventricular fibrillation. Analysis of arrhythmia scoring system-derived values for the collective ventricular ectopy experienced during IR is presented in Fig. 1B. Between-group analysis revealed that all IR treatments exhibited more ventricular arrhythmias ($P \leq 0.001$).

Table 1. Animal number and body weight

<table>
<thead>
<tr>
<th>Group</th>
<th>Number</th>
<th>Body Weight, g</th>
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<tbody>
<tr>
<td>Sham</td>
<td>11</td>
<td>363 ± 10.5</td>
</tr>
<tr>
<td>Sed</td>
<td>17</td>
<td>346 ± 9.9</td>
</tr>
<tr>
<td>Ex</td>
<td>18</td>
<td>340 ± 5.6</td>
</tr>
<tr>
<td>Ex5HD</td>
<td>18</td>
<td>339 ± 6.6</td>
</tr>
<tr>
<td>ExHMR</td>
<td>18</td>
<td>328 ± 5.6</td>
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Values are means ± SE. Sham, surgery without ischemia; Sed, sedentary; Ex, exercised placebo; Ex5HD, Ex 5-hydroxydecanoate mitochondrial ATP-sensitive potassium (K\text{ATP}) channel inhibitor; ExHMR, Ex HMR1098 sarcolemmal K\text{ATP} channel inhibitor.

Fig. 1. Post-ischemia reperfusion (IR) cardiac-outcome variables. A: quantification of percent area at risk (%AAR) for myocardial infarction. Sham, surgery without ischemia; Sed, sedentary; Ex, exercised placebo; Ex5HD, Ex 5-hydroxydecanoate mitochondrial ATP-sensitive potassium (K\text{ATP}) channel inhibitor; ExHMR, Ex HMR1098 sarcolemmal K\text{ATP} channel inhibitor. B: ventricular arrhythmia index scores encompassing preventricular contractions, ventricular tachycardia, and ventricular fibrillation. C: blue = perfused; pink = ischemic viable; white = ischemic necrotic. Values represent mean nonperfused tissue area/total cross-sectional area. Note that Sham hearts received Evan’s blue dye moments before excision as a means of quantifying the region that would have been ischemic. D: infarction/AAR following Sham and IR surgeries. Data are expressed as percentages ± SE. *Significantly different from Sham; †significantly different from Ex5HD; $P < 0.05$. 

J Appl Physiol • doi:10.1152/japplphysiol.00957.2011 • www.jappl.org
than Sham; however, no differences were present between Sed and Ex (Ex, Ex5HD, and ExHMR) IR treatments.

Post-IR myocardial necrosis, apoptosis, and autophagic activity. As an index of post-IR tissue necrosis, TTC staining was quantified as mean infarct area/AAR as presented in Fig. 1C with representative images. Infarcted area was observed in all hearts exposed to IR but not in Sham hearts. Significant between-group differences (P ≤ 0.001) in tissue necrosis were observed within treatments receiving IR. Post hoc analysis revealed that Sed (P = 0.004) and ExHMR (P = 0.002) IR treatments exhibited more ventricular tissue death compared with Sham. No differences in tissue death were noted between Sham and Ex (P = 0.369) and Ex5HD (P = 0.527). Significant differences also existed for mean TTC area/AAR between Ex5HD and ExHMR (P = 0.044), whereas Ex5HD-Sed means were not significantly different (P = 0.069). These findings suggest that the sarc KATP channel mediates exercise-induced cardioprotection against post-IR tissue death.

Apoptosis was determined by the presence of TUNEL-positive nuclei. Representative Sham and ischemic TUNEL images are presented in Fig. 2A with mean responses in Fig. 2B. Data analysis reveals between-group differences (P = 0.037), with post hoc analysis indicating that mean Sed TUNEL values were higher than Sham (P = 0.029). TUNEL values between Ex5HD and Sham did not reach statistical significance (P = 0.177). Representative Bax/Bcl2 blots and β-actin-normalized mean responses are shown in Fig. 2, C and D, respectively. Between-group differences existed for Bax/Bcl2 ratios (P = 0.020). A significant elevation in the Bax/Bcl2 ratio was observed post-IR in the ischemic ventricular tissue from Sham hearts. Bax/Bcl2 ratios were unaltered in the exercised treatments, with the exception that perfused ventricular tissue from Ex5HD animals was lower than Sham.

Normalized means and representative blots for Beclin 1, LC3B ratios, and P62 β-actin are shown in Fig. 3, A–C, respectively. Western blot analyses of autophagic protein abundance in ischemic and perfused ventricular tissues revealed that significant group differences were present for Beclin 1 (P ≤ 0.001), LC3B (P = 0.020) ratios, and P62 (P ≤ 0.001). Post hoc analysis indicates that cardiac levels of Beclin 1 were lower post-IR in the ischemic myocardia from Sed (P = 0.010), Ex5HD (P = 0.013), and ExHMR (P = 0.003), whereas Ex values were similar to Sham (P = 0.393). Within-group (perfused-ischemic) Beclin 1 differences existed for Sed (P = 0.011), Ex5HD (P = 0.017), and ExHMR (P = 0.005) treatments. Compared with Sham, between-group differences for LC3Bl/2/LC3B ratios in ischemic cardiac tissues were lower in Sed (P = 0.049) and ExHMR (P = 0.013) treatments. Analysis of cardiac P62 content from ischemic ventricles revealed ExHMR-Sham between-group differences (P = 0.003), whereas ExHMR perfused-ischemic within-group differences were also present (P = 0.007; Fig. 3C). Although numerical trends were similar to previously mentioned autophagy measures, between-group differences did not exist for cardiac levels of Atg5 (P = 0.161), Atg7 (P = 0.328), and Atg12 (P = 0.332; data not shown).

Myocardial antioxidant enzyme activity in perfused and ischemic tissue. Endogenous antioxidant enzyme activities, evaluated in both ischemic and perfused tissue exposed to IR, are presented in Fig. 4A–C for MnSOD, CAT, and GPx, respectively. Analysis of SOD enzyme activity did not reveal group differences for total SOD (P = 0.095) and CuZnSOD (P = 0.838; data not shown). In contrast, between-group differences did exist for MnSOD enzyme activity (P ≤ 0.001). Post hoc analysis indicated higher MnSOD activity in all three exercised treatments compared with Sham. In particular, MnSOD activities were elevated in perfused tissues of all exercise groups but were increased in the ischemic tissues of Ex and ExHMR only (Ex5HD, P = 0.089). Analysis of cardiac CAT enzyme activity indicated mean group differences (P = 0.002). Specifically, elevated CAT levels were observed in Sed ischemic tissues in addition to ischemic and perfused tissues from Ex and ExHMR groups. Within-group CAT differences existed for Sed hearts only (P = 0.012). Between-group differences were also observed for cardiac GPx enzyme activity (P = 0.022). Post hoc analysis indicated statistically significant drops, compared with Sham, for Sed, Ex, and ExHMR treatments. Within-group GPx enzyme activity differences were observed for Sed hearts only (P = 0.009).

Myocardial oxidative stress following IR. Myocardial oxidative damage was quantified by protein carbonyl content measured in ischemic and perfused ventricular tissues (Fig. 5). Compared with Sham, analyses reveal significant between-group differences (P ≤ 0.001) in post-IR ischemic tissues from Sed and Ex5HD groups only.

DISCUSSION

Summary of principal findings. A short-term exercise regimen (days) is cardioprotective against IR injury (4, 5, 7, 8, 10, 12, 15, 16, 18, 19, 21, 23, 24, 27–29, 33, 35, 37, 38, 40–42), although the cellular mechanisms responsible for this protection are not fully understood. We tested the hypothesis that exercise-induced cardioprotection against an extended-duration IR insult is dependent on both mito KATP and sarc KATP channel activity. This approach is novel in that the mechanisms of exercise preconditioning evolve as ischemic duration extended from 20 to 50 min. Moreover, these data represent a comprehensive examination of post-IR outcomes in hearts from Ex and Sed animals. In contrast to previous examination of shorter IR periods (38), current findings reveal that exercise did not prevent IR-induced ventricular arrhythmias experienced during the 50-min ischemia, 120-min reperfusion challenge. Post-IR evaluation of tissue death confirms that exercise-induced cardioprotection against post-IR necrosis involves both mito KATP and sarc KATP channel activity, whereas the mito KATP channel is not essential. In contrast, protection against post-IR apoptosis was not reliant on either mito KATP or sarc KATP channel activity. Findings do not indicate whether protective outcomes in the exercised hearts were associated with KATP channel alterations in redox balance. Novel examination of tissue autophagy biomarkers in both ischemic and perfused tissue suggests that cardioprotection afforded by short-term exercise may include preservation of basal autophagy during an extended IR challenge.

Exercise-induced cardioprotection against long-duration IR. The current study rationale was founded on the fact that known mechanisms of exercise-induced cardioprotection are specific to the type of IR injury experienced. Within the context of known mechanisms responsible for antiarrhythmic protection due to exercise, MnSOD (21) and mito KATP (38) are independently responsible for exercise-induced prevention of ventricular arrhythmias following a short-duration IR chal-
In response to longer-duration IR, MnSOD (18), endogenous opioids (16), preserved calcium handling (18), and sarc KATP channel activity (7) mediate protection against tissue death. Currently unknown is whether previously observed protection against post-IR apoptotic tissue death in exercised hearts (18, 35, 37) is mediated by either the mito or sarc KATP channel. The IR durations (50-min ischemia, 120-min reperfusion) were chosen to induce apoptosis (18, 35, 37). Given the multifaceted nature of exercise-mediated protection, future investigations should investigate complimentary protection due to sympathetic signaling, tissue temperature, and ionic disturbances among others.

Findings from this investigation reveal for the first time that the mito K$_{ATP}$ channel-mediated protection against ECG abnormalities is lost when IR duration is extended to 50-min ischemia, 120-min reperfusion. Examination of post-IR ECG tracings in addition to necrotic, apoptotic, and autophagic outcomes was performed as a novel, comprehensive assess-
Fig. 3. Ventricular tissue autophagy biomarkers. Representative images and mean responses are presented for cardiac levels of (A) Beclin 1, (B) microtubule-associated protein-1 light-chain 3B (LC3BII/LC3BI) ratios, and (C) P62. Open bars are perfused; closed bars are ischemic or nonperfused (Sham). Data represent semiquantitative analysis of normalized means ± SE. *Significant difference from Sham; #significant difference from perfused; P < 0.05.

Fig. 4. Endogenous antioxidant enzyme activity in ischemic and perfused ventricular tissues. A: manganese (Mn) SOD activity. B: catalase activity. C: glutathione peroxidase (GPX) activity. Open bars are perfused; closed bars are ischemic or nonperfused (Sham). Data are means ± SE. *Significant difference from Sham; #significant difference from perfused; P < 0.05.

The time-dependent nature of IR injury is well understood and has been reviewed previously (34). ECG abnormalities are among the first pathological abnormalities in the first moments of IR. We previ-
usually used a 20-min ischemic insult (30-min reperfusion) and observed that the mito K$_{ATP}$ channel was essential for exercise-induced cardioprotection against arrhythmia generation (38). Tissue death is thought to occur after ~20 continuous min of ischemia (34). The loss of antiarrhythmic protection in exercised hearts suggests that the mito K$_{ATP}$ channel mediates a window of antiarrhythmic protection, which is lost between 20 and 50 min of continuous ischemia.

In contrast to arrhythmia generation, exercise does mediate antinecrotic tissue death following our extended-duration IR protocol. This outcome was assessed by TTC staining and confirms previous findings that the sarc K$_{ATP}$ channel is essential for antinecrotic protection in the exercised heart (7). Several previous studies have demonstrated that short-term exercise elicits protection against post-IR apoptosis in ventricular tissue; however, the protective mediators are currently unknown (18, 35, 37). TUNEL histological sections and Bax/Bcl2 protein content in ischemic and perfused tissue confirm these previous findings. Exercised hearts receiving pharmacologic inhibitors for the mito K$_{ATP}$ channel and sarc K$_{ATP}$ channel remained protected against post-IR apoptotic activity, suggesting that neither ion channel mediates antiapoptotic protection in our short-term exercise model. Treatment-outcome differences between TTC staining and TUNEL assays help to alleviate concerns that TUNEL-positive nuclei may represent necrotic tissue death rather than apoptosis. These data provide new insight into the dynamics of multifaceted cardioprotection afforded by exercise against an in vivo IR insult.

Follow-up experiments in the current study were performed to 1) better understand the post-IR biochemical environments of exercised and sedentary hearts and 2) to ground the current findings to existing research. In regard to post-IR biochemical events, which may impact necrotic and apoptotic outcomes, we chose to investigate several biomarkers for cellular autophagy in perfused and ischemic ventricular sections. Autophagic processes involve lysosomal degradation of cellular constituents. Basal autophagic is increased in the 24–48 h following pathologic events such as IR. Preservation of autophagy in the hours following IR is held to be advantageous in that rapid clearing of cellular debris will facilitate improved recovery. In contrast, large-scale autophagic responses in the days following IR contribute to pathology (26). Currently, we used Western blot to examine the acute autophagic response for several early (Beclin 1 and LC3B) and late cascade autophagy biomarkers (P62). We observed a significant drop in autophagic protein content within the ischemic ventricles of sedentary hearts. By comparison, these protein levels were maintained in ischemic ventricles from exercised animals. This post-IR outcome may reflect a drop in basal autophagy in the ischemic myocardium of sedentary hearts. Maintenance of basal autophagy within exercised hearts in the hours immediately following an ischemic event may represent a novel means of exercise-induced cardioprotection against ischemic injury. Further research is needed before this outcome can be determined conclusively; however, previous findings support this rationale in nonexercise experimental models (26). Given the fact that acute exercise (32), as well as extended exercise training (11), potentiates autophagy in cardiac tissue, future study should examine autophagy and post-IR outcomes in the exercised heart. Further investigation should also examine ventricular tissues at time points 24–48 h post-IR to get a sense of whether exercise inhibits the compensatory rise in pathologic autophagy, which occurs in the days following IR.

Examination of both ischemic and perfused tissues from post-IR hearts has been a simple but important step in furthering our understanding of exercise, cardioprotection, and redox control. Our current finding that protein carbonyl content is elevated in ischemic tissues of sedentary and mito K$_{ATP}$ channel-inhibited animals confirms our previous work with a shorter-duration IR insult (38). In that regard, well-described elevations in MnSOD activity of unstressed exercise hearts (18, 21, 29, 37, 38) appear to have been maintained in hearts receiving an extended-duration IR challenge. CAT activity was increased in ventricular tissues from Ex, ExHMR, and ischemic tissue from sedentary hearts. GPx activity contrasted this finding with significant decreases in ischemic tissues from Sed, Ex, and ExHMR. To our knowledge, this is only the second study to investigate these redox biomarkers in ischemic and perfused tissue from exercised hearts. Further research is needed to better understand whether these redox perturbations, due to exercise and IR, directly impact the cardiac-outcome variables examined in this study. Collectively, our findings extend upon existing data and indicate that redox balance is better preserved in the post-IR heart of exercised animals (21, 27, 29, 37, 38, 40).

**Study limitations.** We used short-term exercise to investigate endogenous mechanisms of cardioprotection against IR injury. The short-term exercise stimulus has several advantages over longer-duration exercise training in that reductionist investigation of protective mediators is not confounded by ventricular remodeling or improved vascularization (34). Previous work, moreover, has demonstrated that the magnitude of IR-injury resistance is similar between short-term exercise training and a longer exercise regimen (15, 33). As such, short-term exercise can be used experimentally to understand endogenous mechanism protection. IR was performed 24 h following the final exercise session, as this time window falls within the 9-day protective time frame identified previously (28). Since unstressed apoptotic indices were not examined currently, we cannot account for the potential that elevated apoptotic mediators may have been altered in exercised hearts as observed previously (39). Nonetheless, examination of perfused and ischemic ventricular tissues was performed as a means of controlling for basal alterations in apoptotic biomarkers. Given the link between post-IR tissue death and cardiac function,
future investigations may benefit from the inclusion of left ventricular-developed pressure data. These measures can be obtained when the arterial cannula is advanced into the left ventricle.

The pharmacologic inhibitors 5HD and HMR1098 were used currently to examine whether the mito or sarc K\textsubscript{ATP} channel mediates IR-injury resistance. Inherent to all physiologic experiments using pharmacologic inhibitors, the current drugs have undeniable limitations, including the potential for nonspecific metabolic effects (2, 22). Nonetheless, these inhibitors were chosen for the following reasons. These are the only widely used inhibitors thought to be selective for the mito or sarc K\textsubscript{ATP} channel (7, 38). Knockout animals for components of the cardiac K\textsubscript{ATP} channel are intolerant of exercise, making them unsuitable for use in the current study (45). Moreover, knockout models are equally unsuitable in that exercise is likely to activate protection through opening of constitutively expressed K\textsubscript{ATP} channels.

Conclusions. Exercise-induced cardioprotection against IR injury is an emerging subfield in physiologic science. Exercise-induced cardioprotection has gained recent attention in that the experimental model is pragmatic and sustainable and may prove beneficial in understanding new approaches in treating ischemic heart disease [reviewed in (9, 34, 36)]. The mechanisms responsible for the protection derived by exercise are only partially understood. The current investigation provides novel understanding of the mito and sarc K\textsubscript{ATP} channels relative to several cardiac outcomes in exercised hearts. First, mito K\textsubscript{ATP} channel-mediated antiarrhythmic protection is lost when ischemic durations are extended to 50 min, as in the current study. Whereas the sarc K\textsubscript{ATP} channel was confirmed to provide antinocrotic protection in the exercised heart, it was not responsible for antiaapoptotic protection due to exercise. Finally, exercise appears to maintain basal autophagy levels in the hours immediately following IR, although this response is incompletely understood. Further research is also needed to understand how redox changes in exercised hearts translate to improved outcomes during IR injury. Collectively, these findings reveal new insight that the mechanisms of exercise-induced cardioprotection appear to change in a time-dependent fashion. This multifaceted protection in exercised hearts is important in meeting the evolving pathology that is IR injury.

ACKNOWLEDGMENTS

The authors thank Dr. R. Andrew Shanely for his technical assistance in preparation of the study design.

GRANTS

Support for these experiments was provided by grants from the Appalachian State University Research Council (J. C. Quindry) and the National Heart, Lung, and Blood Institute (J. C. Quindry; HL087256). HMR1098 was a generous gift of Dr. Heinz Golgolein (Adventis Pharma, Germany).

DISCLOSURES

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

AUTHOR CONTRIBUTIONS

Author contributions: J.C.Q. conception and design of research; J.C.Q., L.M., G.M., B.K., J.M.I., M.L., Z.U., G.N., and R.A. interpreted results of experiments; J.C.Q. prepared figures; J.C.Q. drafted manuscript; J.C.Q. edited and revised manuscript; J.C.Q. approved final version of manuscript.

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