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ACTIVITY ATTENUATES SKELETAL MUSCLE FIBER DAMAGE AFTER ISCHEMIA AND REPERFUSION

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ABSTRACT: *Introduction:* In this investigation we aimed to determine whether: (1) physical activity protects rat skeletal muscle from ischemia/reperfusion (I/R) injury; and (2) continued activity after I/R improves the rate of healing. *Methods:* Rats were divided into sedentary or active (voluntary wheel running) groups. Active rats ran for 4 weeks before I/R or 4 weeks before plus 4 weeks after I/R. *Results:* Activity before I/R resulted in 73.2% less muscle damage (Evans blue dye inclusion). Sedentary and active rats had a similar decline in neural-evoked (~99%) and directly stimulated (~70%) *in vivo* muscle torque, and a similar reduction in junctophilin 1. Active rats produced 19% and 15% greater neural-evoked torque compared with sedentary rats at 14 and 28 days postinjury, respectively, although the rate of recovery appeared similar. *Conclusions:* Activity protects against long-term muscle damage, but not short-term neural injury or excitation-contraction uncoupling. Continued activity neither accelerates nor hinders the rate of functional recovery.

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Extremity injuries are highly prevalent in both military¹ and civilian² trauma, and they often involve skeletal muscle.³ In many cases, prolonged blood flow disruption due to concomitant vascular damage and/or acute compartment syndrome induces tissue ischemia, and subsequent surgical repair promotes reperfusion of the tissue.⁴ Tissue reperfusion is necessary for limb salvage, although free radicals generated during this process further injure vascular, neural, and muscular structures [i.e., ischemia/reperfusion (I/R) injury^{5–7}]. The underlying molecular mechanisms of I/R injury are fairly well understood, and a number of protective agents^{8–10} and

cell therapies^{10–12} have been tested as potential treatments. In considering therapies, however, patient characteristics, including age and activity level, may dictate the severity and rate of recovery after I/R injury.¹³ Surprisingly, the benefits of physical activity in protecting or improving recovery after limb I/R injury have not been tested previously in an animal model.

Numerous studies have demonstrated that aerobic activity confers cardioprotection against I/R injury.^{14,15} Cardioprotective factors include increased collateral circulation, induction of heat shock proteins, and improved antioxidant capacity.¹¹ Interestingly, these factors are also increased in skeletal muscle in response to aerobic activity,^{16–18} suggesting that regular aerobic activity may offer protection against I/R injury in muscle.

The beneficial role of activity in the form of physical rehabilitation after musculoskeletal injuries is well accepted, particularly in the case of sports-related injuries, such as muscle strain or rupture.^{19,20} Clinically, rehabilitation focuses primarily on muscle strengthening and restoring range of motion through stretching.²⁰ A major focus of laboratory research involving rehabilitation has been the role of early locomotor activity.²⁰ Treadmill running has been found to be essential for optimal structural organization after muscle contusion.^{21,22} However, it is unknown whether early locomotor activity improves and/or hastens functional recovery following I/R injury.

There remains much to be studied about the role of physical activity in protection and recovery of skeletal muscle after I/R injury. Here we present an initial investigation into the impact of increased activity (voluntary wheel running) in a rat I/R injury model. Because both the ability and likelihood of engaging in postinjury activity is strongly dependent on whether patients were active before injury,²³ as a first approach, we compared rats that were active before and after I/R injury to rats that were sedentary (normal cage activity). Our overarching questions were: (1) does previous activity protect from injury; and (2) does continuous activity accelerate long-term recovery?

METHODS

Experimental Design. Rats were placed in either “sedentary” or “active” groups. All active rats were

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Abbreviations: ANOVA, analysis of variance; DAPI, 4',6-diamidino-2-phenylindole; EBD, Evans blue dye; EC, excitation-contraction; EDL, extensor digitorum longus; FG, fast glycolytic; FOG, fast oxidative glycolytic; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; H&E, hematoxylin and eosin; I/R, ischemia/reperfusion injury; PGC1 α , peroxisome proliferator-activated receptor gamma coactivator 1 α ; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; TA, tibialis anterior muscle; TBST, Tris-buffered saline plus Tween-20

Key words: exercise; junctophilin 1; muscle injury; rats; trauma

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housed with unrestricted access to a running wheel 4 weeks before I/R injury. Sedentary rats were housed in a standard cage [39.5 cm (w) × 34.6 cm (l) × 21.3 cm (h)]. Two studies were performed. In Study 1, the goal was to determine whether previous activity protected skeletal muscle from I/R injury. To do so, rats from each group were euthanized 4 days postinjury. In Study 2, an additional subset of previously active and sedentary rats continued their respective activity for 28 days postinjury to determine whether continuation of their previous activity increased the rate of functional recovery after I/R injury. Sample sizes for each experiment are described.

Animals. Adult, male, 4-month-old Lewis rats were obtained from colonies at Charles River Laboratories (Wilmington, Massachusetts). This study was conducted in compliance with the Animal Welfare Act, the Implementing Animal Welfare Regulations, and in accordance with the principles of *The Guide for the Care and Use of Laboratory Animals*. All procedures were approved by the institutional animal care and use committee at the U.S. Army Institute of Surgical Research. Rats were housed in a vivarium accredited by the American Association for Assessment and Accreditation of Laboratory Animal Care International. They were housed individually with a 12-hour light/dark cycle and provided with standard rat chow and water *ad libitum*. Active rats were housed with a running wheel (Model 80859; Lafayette Instruments, Lafayette, Indiana). Running distance was monitored constantly and recorded on a personal computer using the hardware and software provided with the system, and the total daily distance run by each rat was determined.

I/R Injury. Anesthetized rats (isoflurane 2%–2.5%) underwent 3 h of pneumatic tourniquet-induced I/R injury at a pressure of 250 mm Hg.²⁴ Ischemia was induced using a unilateral pneumatic tourniquet around the thigh. A commercially available digit cuff (Model DC 1.6; D.E. Hokanson, Inc.) was placed as high as possible on the left hindlimb. While in a supine position, the hindlimb was exsanguinated by elevating it for 5 min before application of tourniquet pressure. Pressure was maintained in the tourniquet using a rapid cuff inflator equipped with a pressure transducer (Model E20; D.E. Hokanson, Inc.). Sustained-release buprenorphine-HCl (ZooPharm, Fort Collins, Colorado) was injected (1.2 mg/kg subcutaneously) for pre-emptive analgesia.

In Vivo Anterior Crural Muscle Functional Assessment. *In vivo* functional assessment of the anterior crural muscles (tibialis anterior, extensor digitorum longus, and extensor hallucis longus)

was done repeatedly at 28 and 2 days preinjury and 4, 14, and 28 days postinjury, using a dual-mode servomotor (Model 305b; Aurora Scientific, Inc.) and methodology we described previously for mice and rats.^{10–12,25–29} During testing, rats were anesthetized (isoflurane 2%–2.5%) and core body temperature was monitored, and maintained at 36°–37°C. Maximal tetanic isometric torque was determined by stimulating the common fibular nerve via percutaneous electrodes using a Grass stimulator (S88) at 150 Hz with a pulse width of 0.1 ms using an optimized voltage (2–8 V). In addition, maximal isometric tetanic torque was determined in subgroups ($n = 4$ per group) of rats via direct muscle stimulation (150 Hz, 0.2-ms pulse, 3–10 V) by inserting electrodes in the proximal and distal portions of the muscle compartment perpendicular to its long axis. Preliminary testing indicated direct stimulation elicited ~90% of neural-stimulated torque in uninjured muscle. A custom program (LabVIEW; National Instruments, Inc.) was used to control the muscle lever systems and to collect, store, and analyze the data.

Evans Blue Dye Assessment of Muscle Fiber Damage. The inclusion of Evans blue dye (EBD) within cells, an index of muscle fiber damage,³⁰ was assessed in subgroups of rats from each treatment group 4 days postinjury ($n = 5$ or 6 per group). Sixteen hours before tissue harvest, rats were injected via tail vein with EBD dissolved in sterile phosphate-buffered saline (10 μ l/g body weight).³¹ Tibialis anterior (TA) muscles were then frozen and sectioned for histological analysis as described previously.²⁵ Cross-sections were probed with wheat germ agglutinin (1:20; Alexa Fluor 488 WG; Invitrogen, Grand Island, New York) and 4',6-diamidino-2-phenylindole (DAPI; 1:100; Invitrogen) to detect muscle fiber boundaries and nuclei, respectively. EBD auto-emits red fluorescent light, and the area positive for EBD was determined in whole muscle cross-sections using ImageJ with methods similar to those described previously.³²

Muscle Fiber Cross-Sectional Area and Centrally Located Nuclei Analyses. Fiber cross-sectional area in TA muscles harvested 28 days postinjury was determined using a custom-written macro in ImageJ (NIH, Bethesda, Maryland), using methodology described by Meyer and Lieber³³ and by our group.^{25,27} Images (10 per muscle) of laminin-probed (1:200; AB11575; Abcam, Cambridge, Massachusetts; and 1:200; Alexa Fluor 488; Invitrogen) sections ($n = 4$ muscles per group; >4000 fibers per group) were analyzed. Only fibers with sizes between 50 and 6000 μ m² and a circularity between 0.3 and 1.0 were included for analysis

Table 1. Anterior crural muscle weights (mg/g body weight).

	Uninjured		Injured	
	Sedentary	Active	Sedentary	Active
4 days postinjury				
EDL	0.37 ± 0.01	0.42 ± 0.01*	0.40 ± 0.01	0.41 ± 0.01
TA	1.42 ± 0.04	1.59 ± 0.01*	1.65 ± 0.12 [†]	1.69 ± 0.12
28 days postinjury				
EDL	0.42 ± 0.02	0.43 ± 0.01	0.40 ± 0.01	0.41 ± 0.01
TA	1.67 ± 0.04	1.78 ± 0.02*	1.61 ± 0.03	1.65 ± 0.03 [†]

Values expressed as mean ± standard error of the mean.

*Significantly different from sedentary ($P < 0.05$).

[†]Significantly different from uninjured ($P < 0.05$).

to remove the measurement of neurovascular bundles, optically fused fibers, and oblique fibers.³³ Fibers on the border of the image were also excluded from analysis.

Hematoxylin-and-eosin (H&E)-stained sections were analyzed for the presence of fibers with centrally located nuclei. Eight to 10 non-overlapping 10× images of a cross-section from the middle of the TA muscle from 5 muscles from each group at 28 days postinjury were analyzed using ImageJ.

Western Blot. Soluble protein was extracted from cross-sections of TA muscles ($n = 5$ muscles per group), as described previously.³⁴ Protein concentrations were determined with the Pierce bicinchoninic protein assay kit (Thermo Scientific). Proteins were resolved by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 20 µg of total protein per sample on 4%–20% Tris-glycine gels (Bio-Rad). Transfer was made onto nitrocellulose membranes, stained with Ponceau S stain to ensure equal loading, and then blocked for 1 h at room temperature in Tris-buffered saline containing 0.05% (v/v) Tween-20 (TBST) and 5% (w/v) nonfat dried milk. Membranes were then incubated overnight at 4°C in TBST containing 5% (w/v) bovine serum albumin and heat shock protein 70 (Enzo Life Sciences), cytochrome c (Invitrogen), junctophilin (Invitrogen), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Millipore) primary antibody diluted 1:1000. Membranes were rinsed 6 times in TBST and then incubated at room temperature for 1 h in TBST and 5% milk containing peroxidase-conjugated goat anti-rabbit secondary antibody diluted 1:3000. Membranes were rinsed 6 times in TBST before exposure to enhanced chemiluminescence reagents (Invitrogen). The membranes were then imaged using the Odyssey Fc system (LI-COR Biosciences, Lincoln, Nebraska).

Quantitative Reverse Transcription-Polymerase Chain Reaction. RNA was isolated from snap-frozen cross sections of TA (50–100 mg) and reverse tran-

scribed to make cDNA. Aliquots (2 µl) of cDNA were amplified with 200 nM forward/reverse primers and SYBR GreenER in triplicate using a thermal cycler system (CFX96; Bio-Rad). Non-template and non-reverse transcriptase controls were run for each reaction. Gene expression was normalized to 18S (housekeeping gene) to determine the change in cycle threshold (Δ CT) value. Expression levels for each mRNA transcript ($n = 4$ per group) were determined by the $2^{-\Delta\Delta CT}$ method by normalizing each group to the uninjured muscle of the sedentary group.²⁵ All primer sets were synthesized using a DNA oligomer design tool (Sigma-Aldrich) (Table 1).

Statistics. Data were analyzed using a variety of analyses of variance (ANOVAs) or independent t -test. Upon finding a significant ANOVA, *post hoc* means comparisons testing was performed using Fisher t -tests. All statistical comparisons were performed using commercially available software (PASW Statistics 18). Statistical significance was determined at $\alpha = 0.05$. All data are presented as mean ± standard error of the mean (SEM).

RESULTS

Voluntary Wheel Running and Body Weight. Rats increased running distance progressively over the 4 weeks before injury and resumed running within the first 24 h post-injury; distances matched preinjury weekly running distances by week 2 postinjury (Fig. 1A). The average total distances run for the 4-week periods before and after injury were 45.3 ± 2.9 km and 43.8 ± 7.2 km, respectively.

The body weights of sedentary and active rats were similar at study initiation (Fig. 1B). Sedentary rats underwent significant weight gain within the first week and, with the exception of week 5 (the week after I/R), continued to gain weight throughout the study. In contrast, the body weights of active rats did not differ markedly from study initiation until the final week of the study, and were significantly lower than weights of sedentary rats throughout the study (Fig. 1B). Due to differences

FIGURE 1. Running distance and body weight of active and sedentary rats before and after I/R injury. **(A)** Average weekly running distance of active rats. **(B)** Average weekly body weight for sedentary and active rats. *Sedentary > active ($P < 0.05$).

in growth curves, morphological and functional indices were normalized to body weight.

Study 1: Protective Effect of Previous Activity.

Muscle Weight. The muscle weights of the injured TA and extensor digitorum longus (EDL) muscles from the sedentary group were significantly greater than those of the corresponding uninjured contralateral muscles ($P = 0.005$ and 0.009 , respectively) (Table 1), indicating injury-induced edema. The uninjured contralateral muscle weights (Table 1) of active rats were significantly greater than those of sedentary rats for both the TA and EDL muscles ($P = 0.03$ and 0.001 , respectively), indicating a training effect on muscle weight.

Muscle Fiber Damage and Tissue Inflammation. Inspection of H&E-stained cross-sections of muscles from sedentary rats revealed marked damage 4 days after I/R injury, characterized necrotic muscle fibers, inflammatory cells, and extra- and intracellular edema (indicated by large round cells) (Fig. 2A). In contrast, the region of damage was visibly smaller in active rats, and there were noticeably fewer signs of edema and presumptive immune cell infiltration (Fig. 2B).

The magnitude of sarcolemmal damage was quantified after EBD injection. Intracellular EBD inclusion constituted only $9.8 \pm 3.8\%$ of TA muscle cross-sectional area in active rats compared with $36.5 \pm 3.3\%$ in sedentary rats ($P < 0.001$; $n = 5$ or 6 per group) (Fig. 2C and D). There was also a notably greatly deposition of extracellular material and mononuclear cellular presence in TA muscles from sedentary rats localized near the region of EBD⁺ fibers (Fig. 2E and F). The regions of damage identified by H&E and EBD inclusion were colocalized in adjacent serial cross-sections and were located primarily in the superficial portion of the TA muscle. Notably, the portions of the muscle not directly involved did not show histological evidence of fiber damage.

To further characterize the inflammatory response evident from histology, pro- and anti-inflammatory cell gene expression was quantified (Fig. 3; $n = 4$ per group). *CCR7* expression (pro-inflammatory) was elevated by ≈ 28 - and 60-fold in sedentary and active rats, respectively. I/R elevated *CD163* (anti-inflammatory/pro-regenerative; M2 macrophage-specific) by ≈ 50 -fold and ≈ 150 -fold for sedentary and active rats, respectively; both macrophage markers were significantly different between groups ($P = 0.02$ – 0.03). However, *TGF- β 1* (pro-fibrotic) was similarly and significantly elevated in injured muscles from sedentary and active rats. Collectively, these results indicate a greater relative anti-inflammatory/pro-regenerative response in injured muscle from active rats.

In Vivo Isometric Torque Production. Before injury, sedentary and active groups produced similar torque values (74.4 ± 1.8 and 77.3 ± 2.5 Nmm/kg, respectively; $P = 0.394$; $n = 4$ per group). At day 4 postinjury, muscles from both groups displayed nearly complete loss of neural-activated isometric torque (Fig. 4A), as demonstrated previously.³⁵ In a subset of sedentary and active rats, the anterior crural muscles were stimulated directly, bypassing upstream components of the excitation–contraction (EC) coupling process (i.e., nerve and neuromuscular junction), to estimate the contributions of neural and muscle injuries to these functional deficits (Fig. 4A). Direct muscle stimulation elicited similar maximal isometric torque values of 17.4 ± 6.5 and 25.6 ± 4.7 Nmm/kg for sedentary and active rats ($P = 0.354$), respectively, indicating that $\sim 23\%$ – 33% of the strength deficit was attributed to disruption of neural activation of the muscle (e.g., neural injury or creation of abjunctional muscle fiber stumps^{36,37}). These data therefore suggest that the majority ($\sim 67\%$ – 77%) of the neuromuscular strength deficit was due to either the loss of force-bearing proteins^{38–40} or disruption of voltage-induced sarcoplasmic reticulum Ca^{2+} release.^{41–43}

FIGURE 2. Representative histology of injured TA muscle cross-sections at 4 days postinjury in sedentary and active rats. (**A, B**) Cross-sections were stained with hematoxylin and eosin (H&E). (**C, D**) Evans blue dye (EBD; red fluorescence) inclusion in damaged muscle fibers after intraperitoneal injection. (**E, F**) EBD inclusion (red) merged with cross-sections probed with wheat germ agglutinin (green) and nuclear stain (DAPI; blue). Scale bars = 100 μ m.

In both the sedentary and active groups, the functional deficits attributed to muscle injury were disproportionately large compared with the damaged area (Fig. 2), suggesting disruption of EC coupling⁴² as a primary determinant of strength loss in the remaining intact muscle fibers. Accordingly, junctophilin 1, a protein crucial to EC coupling in skeletal muscle,⁴⁴ which is susceptible to mechanical²⁹ and oxidative stress,⁴⁵ underwent a similar reduction in injured sedentary (−56%) and active (−50%) TA muscles (Fig. 4B). Collectively, these findings indicate that, although activity protected TA muscles from sarcolemmal damage, both neural disruption and EC uncoupling were not attenuated, and therefore a similar and nearly complete loss of neural-stimulated strength was observed between sedentary and active rats (Fig. 4C).

Increased Aerobic Phenotype. We have observed previously that muscles with greater oxidative capacity are less susceptible to injury,⁴⁶ so we sought to determine whether the protective effect

of previous activity was due to an elevated aerobic phenotype in the TA muscle. To do so, cytochrome c protein content was measured in uninjured and injured TA muscles from sedentary and active rats 4 days postinjury ($n = 5$ per group). No significant differences were observed among groups (see Fig. S1A in Supplementary Material, available online). Peroxisome proliferator-activated receptor gamma coactivator 1 α (PGC1 α) expression, which is associated with mitochondrial biogenesis, was also measured in TA muscles 4 days postinjury ($n = 4$ per group). In all injured muscles, PGC1 α was downregulated, but expression tended to be upregulated (by 1.75-fold compared with sedentary control) in uninjured active muscles (see Fig. S1B in Supplementary Material).

Study 2: Effect of Continued Activity on Recovery from I/R Injury.

Muscle Weight. At 28 days postinjury, the impact of activity on muscle weight was evident by significantly greater weight (mg/g) in the uninjured TA muscles from active vs. sedentary

FIGURE 3. Gene expression for inflammatory markers at 4 days postinjury. (A) *CCR7* (pro-inflammatory), (B) *CD163* (anti-inflammatory), and (C) *TGF-β1* (anti-inflammatory) gene expressions were measured using qRT-PCR in sedentary and active control (uninjured) and injured TA muscles. * $\neq$ Active control, ** $\neq$ all other groups ($P < 0.05$).

animals ($P = 0.002$) (Table 1). The weight of injured TA muscle from the active group was significantly lower than that of the corresponding uninjured contralateral muscle ($P = 0.014$) (Table 1). In contrast to the TA, there was no significant impact of injury or activity on the weights of the EDL muscle.

In Vivo Isometric Torque Production. Anterior crural muscle maximal isometric torque of the injured limb was measured repeatedly before activity (-28 days), before injury (0 day), and at 4, 14, and 28 days postinjury while continuing activity or remaining sedentary ($n = 5$ per group; Fig. 5). Before activity (sedentary vs. active: 67.1 ± 2.5 vs. 68.6 ± 1.7 Nmm/kg; $P = 0.653$) and injury (sedentary vs. active: 73.0 ± 2.0 vs. 71.6 ± 2.2 Nmm/kg;

$P = 0.569$), maximal isometric torque was similar between the groups. In addition, as noted previously, at 4 days postinjury, all rats exhibited a nearly complete loss of neural-evoked torque. At 14 days postinjury, active rats had a significantly lower torque deficit than sedentary rats (33.8 ± 2.21 vs. $42.5 \pm 2.24\%$; $P = 0.02$). By 28 days postinjury, active rats had reached preinjury values, whereas sedentary rats still exhibited an $18.2 \pm 2.4\%$ torque deficit ($P = 0.02$).

Tissue Regeneration and Morphology. Twenty-eight days postinjury, semi-quantitative analysis of muscle fibers with centrally located nuclei in TA muscles from each group was performed as an index of muscle fiber regeneration (see Methods for sampling details). TA muscles had a similar number of fibers with centrally located nuclei, regardless of activity group (see Fig. S2 in Supplementary Material). In addition, muscle fiber area was quantified in injured TA muscles from sedentary and active groups. Average fiber area was significantly smaller in sedentary than active rats at 28 days postinjury (see Fig. S3 in Supplementary Material).

DISCUSSION

Anecdotal, there is a strong sense that active individuals can tolerate muscle injury better than sedentary individuals, but no experimental evidence supports this contention. Here we have presented clear evidence that continuous activity before I/R injury reduces the magnitude of muscle fiber damage acutely after injury. Furthermore, this activity-induced attenuation of tissue damage likely contributes to accelerated recovery of strength after injury.

Strength deficits after I/R are attributed collectively to nerve and muscle fiber injury.^{35,47} Injury to skeletal muscle fibers after I/R is likely to manifest strength deficits via the loss of force-bearing proteins and disruption of voltage-induced Ca^{2+} release from the sarcoplasmic reticulum (SR).^{8,38,47} In addition, muscle fiber lesions can also create temporary denervated abjunctional fiber segments.^{36,37} Whereas I/R injury-induced muscle fiber damage was reduced in active rats, the activity did not attenuate the early loss of muscle strength. Virtually complete loss of neural-evoked torque production was observed 4 days postinjury regardless of activity (Figs. 4 and 5). As a partial explanation for this discrepancy, we estimate that $\sim 30\%$ of the strength deficit was due strictly to disruption of neural activation of functional muscle fibers,^{48–50} to include abjunctional fiber segments^{36,37}; however, the nature of neural disruption was not specifically investigated. The remaining $\sim 70\%$ of the observed neural-evoked torque deficit was attributed to

FIGURE 4. Acute functional deficits after I/R injury are multifactorial. **(A)** Neural and direct strength assessment of the anterior crural muscles was performed before and after I/R injury. Isometric torque was similar between groups in all conditions. **(B)** Junctophilin 1 protein content was measured in control (uninjured) and injured TA muscles from active and sedentary rats. * $\neq$ control muscles ($P < 0.05$). **(C)** Diagram of the contribution of failing elements to neuromuscular torque production in sedentary and active rats. Fiber damage was quantified from EBD inclusion. Neural damage was calculated as the difference between neural-stimulated and directly stimulated functional deficits. EC coupling contribution was considered the remaining functional deficit. Despite significantly smaller muscle damage evidenced by EBD inclusion, concomitant neural injury and EC coupling disruption resulted in a similar and nearly complete loss of neural-stimulated strength 4 days postinjury in sedentary and active rats.

either a loss of force-bearing proteins or EC coupling failure at or downstream from the sarcolemma. Given that active rats had significantly less muscle fiber damage (Fig. 2), a likely explanation for the similar and nearly complete torque deficit between groups is EC coupling failure (i.e., disrupted SR Ca^{++} release) in the remaining intact muscle fibers. Furthermore, junctophilin 1 protein content was reduced by $\sim 50\%$ in the injured muscles from sedentary and active rats (Fig. 4). Junctophilin 1 is critical to voltage-induced Ca^{++} release and has been shown to be damaged in response to mechanical²⁹ and Ca^{++} -dependent proteolysis secondary to oxidative stress.⁴⁵ These findings are consistent with the idea that EC coupling is a positive response to muscle injury^{29,51} that either protects muscle from further injury or, possibly, conserves energy for its repair.

Muscle fiber damage was confined primarily to the superficial regions of the muscles, regardless of treatment (Fig. 2A–F). Anatomically, the superficial portion is composed primarily of fast glycolytic

(FG) fibers, whereas the deep portion is composed predominantly of fast oxidative glycolytic (FOG) fibers.⁵² The sensitivity of skeletal muscle to I/R is a function of fiber type composition; that is, FOG fibers are less sensitive than FG.⁴⁶ The observation that the area of injury was smaller in this region in response to activity suggests that protection was conferred upon the region corresponding to predominantly FG fibers. However, if these fibers had been activated substantially during activity, we would have expected evidence of a training effect, such as increased mitochondrial biogenesis. Although we saw evidence of this at the transcriptional level (PGC1 α), we found no increase in the mitochondrial marker cytochrome c. Activity resulted in an enhanced anti-inflammatory/pro-regenerative (M2) response in injured muscle, as indicated by expression of CD163 (Fig. 3), which is consistent with other reports.⁵³ The activity-induced enhanced anti-inflammatory response may have played a role in mitigating I/R-induced muscle damage.

FIGURE 5. Anterior crural muscle isometric strength before and after I/R injury in sedentary and active rats. **(A)** Isometric torque (maximal tetanic stimulation; 400-ms train, 0.1-ms pulse, 150 Hz) was measured repeatedly in each rat and normalized to preinjury values (0 d). *Sedentary $\neq$ active ($P < 0.05$).

Restoration of physical activity after neuromuscular and musculoskeletal injuries is intuitively and experimentally understood to be beneficial to muscle healing and maintenance of muscle mass.^{19,54} Clinically, injured patients often experience disuse and immobilization. Previous works that form the foundation for our study have shown that countermeasures to prolonged disuse are undeniably beneficial to muscle regeneration after crush and contusion injury.^{21,22,55–59} The many benefits include improved muscle fiber repair and regeneration, reduced fibrosis, enhanced early revascularization, and re-establishment of normal muscle mechanical properties. Of note, reversal of the catabolic state promoted by prolonged immobilization can require only a minimal amount of activity (i.e., normal cage activity).^{21,60–62} As an extension of these findings, in this study we assessed whether sedentary (normal cage activity) and active (voluntary wheel running) rats recovered from I/R injury in a similar manner. Activity resulted in significantly greater strength at both 14 and 28 days post-I/R (Fig. 5). In fact, muscle strength had recovered to pre-I/R levels statistically by the later time-point, and the greater functional recovery in active rats was consistent with significantly greater mean fiber area (see Fig. S3 in Supplementary Material). However, despite the difference in strength between active and sedentary groups, the actual rate of strength recovery between 14 and 28 days was essentially the same, at 24%–28%. Therefore, the greater recovery of strength after injury in active rats was likely due to attenuation of the initial injury rather than an accelerated rate of healing. These findings broadly highlight that, although greater activity may be needed to protect

skeletal muscle from I/R injury (i.e., sedentary vs. active), improved regeneration after injury may only require a low level of activity (i.e., immobilized vs. normal cage activity/sedentary⁶²).

In conclusion, the key finding of our study is that continuous physical activity confers protection against I/R injury-induced muscle fiber damage in rats. The results do not demonstrate a strong therapeutic benefit to the early resumption of activity in comparison to the sedentary condition, although the literature suggests that benefits can be realized when compared with immobilization.^{55,61,62} Instead, the protective effect of activity resulted in accelerated functional recovery. The results suggest a possible role of immune modulation in active rats, although the mechanism(s) responsible for the protective effects of activity remain to be elucidated.

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REFERENCES

- Owens BD, Kragh JF Jr, Macaitis J, Svoboda SJ, Wenke JC. Characterization of extremity wounds in Operation Iraqi Freedom and Operation Enduring Freedom. *J Orthop Trauma* 2007;21:254–257.
- Sleet DA, Moffett DB. Framing the problem: injuries and public health. *Fam Commun Health* 2009;32:88–97.
- Owens BD, Kragh JF Jr, Wenke JC, Macaitis J, Wade CE, Holcomb JB. Combat wounds in Operation Iraqi Freedom and Operation Enduring Freedom. *J Trauma* 2008;64:295–299.
- Percival TJ, Rasmussen TE. Reperfusion strategies in the management of extremity vascular injury with ischaemia. *Br J Surg* 2012; 99(suppl 10):66–74.
- Percival TJ, White JM, Ricci MA. Compartment syndrome in the setting of vascular injury. *Perspect Vasc Surg Endovasc Ther* 2011;23: 119–124.
- Gifford SM, Eliason JL, Clouse WD, Spencer JR, Burkhardt GE, Propper BW, *et al.* Early versus delayed restoration of flow with temporary vascular shunt reduces circulating markers of injury in a porcine model. *J Trauma* 2009;67:259–265.
- Fox CJ, Gillespie DL, Cox ED, Mehta SG, Kragh JF Jr, Salinas J, *et al.* The effectiveness of a damage control resuscitation strategy for vascular injury in a combat support hospital: results of a case control study. *J Trauma* 2008;64(suppl):S99–107.
- Ward CL, Corona BT, Yoo JJ, Harrison BS, Christ GJ. Oxygen generating biomaterials preserve skeletal muscle homeostasis under hypoxic and ischemic conditions. *PLoS One* 2013;8:e72485.
- Gillani S, Cao J, Suzuki T, Hak DJ. The effect of ischemia reperfusion injury on skeletal muscle. *Injury* 2012;43:670–675.
- Corona BT, Rathbone CR. Accelerated functional recovery after skeletal muscle ischemia-reperfusion injury using freshly isolated bone marrow cells. *J Surg Res* 2014;188:100–109.
- Chen XK, Rathbone CR, Walters TJ. Treatment of tourniquet-induced ischemia reperfusion injury with muscle progenitor cells. *J Surg Res* 2011;170:e65–73.
- Corona BT, Wenke JC, Walters TJ, Rathbone CR. Intramuscular transplantation and survival of freshly isolated bone marrow cells following skeletal muscle ischemia-reperfusion injury. *J Trauma Acute Care Surg* 2013;75(suppl 2):S142–149.
- Zhou Y, Lovell D, Betha M, Wang Z, Christ GJ, Soker S, *et al.* Age-dependent changes cooperatively impact skeletal muscle regeneration after compartment syndrome injury. *Am J Pathol* 2014;184: 2225–2236.
- Starnes JW, Taylor RP. Exercise-induced cardioprotection: endogenous mechanisms. *Med Sci Sports Exerc* 2007;39:1537–1543.
- Powers SK, Lennon SL, Quindry J, Mehta JL. Exercise and cardioprotection. *Curr Opin Cardiol* 2002;17:495–502.
- Huey KA, Meador BM. Contribution of IL-6 to the Hsp72, Hsp25, and alphaB-crystallin [corrected] responses to inflammation and exercise training in mouse skeletal and cardiac muscle. *J Appl Physiol* 2008;105:1830–1836.
- Powers SK, Ji LL, Leeuwenburgh C. Exercise training-induced alterations in skeletal muscle antioxidant capacity: a brief review. *Med Sci Sports Exerc* 1999;31:987–997.

18. Vemuganti GK, Mandal AK. Subepithelial corneal amyloid deposits in a case of congenital glaucoma: a case report. *Cornea* 2002;21:315–317.
19. Jarvinen TA, Jarvinen TL, Kaariainen M, Aarimaa V, Vaitinen S, Kalimo H, *et al.* Muscle injuries: optimising recovery. *Best Pract Res Clin Rheumatol* 2007;21:317–331.
20. Jarvinen TA, Jarvinen TL, Kaariainen M, Kalimo H, Jarvinen M. Muscle injuries: biology and treatment. *Am J Sports Med* 2005;33:745–764.
21. Jarvinen MJ, Lehto MU. The effects of early mobilisation and immobilisation on the healing process following muscle injuries. *Sports Med* 1993;15:78–89.
22. Lehto M, Duance VC, Restall D. Collagen and fibronectin in a healing skeletal muscle injury. An immunohistological study of the effects of physical activity on the repair of injured gastrocnemius muscle in the rat. *J Bone Joint Surg Br* 1985;67:820–828.
23. Jack K, McLean SM, Moffett JK, Gardiner E. Barriers to treatment adherence in physiotherapy outpatient clinics: a systematic review. *Man Ther* 2010;15:220–228.
24. Walters TJ, Mase VJ Jr, Roe JL, Dubick MA, Christy RJ. Poloxamer-188 reduces muscular edema after tourniquet-induced ischemia-reperfusion injury in rats. *J Trauma* 2011;70:1192–1197.
25. Corona BT, Garg K, Ward CL, McDaniel JS, Walters TJ, Rathbone CR. Autologous minced muscle grafts: a tissue engineering therapy for the volumetric loss of skeletal muscle. *Am J Physiol Cell Physiol* 2013;305:C761–775.
26. Corona BT, Ward CL, Baker HB, Walters TJ, Christ GJ. Implantation of in vitro tissue engineered muscle repair constructs and bladder acellular matrices partially restore in vivo skeletal muscle function in a rat model of volumetric muscle loss injury. *Tissue Eng Part A* 2014;20:705–715.
27. Corona BT, Wu X, Ward CL, McDaniel JS, Rathbone CR, Walters TJ. The promotion of a functional fibrosis in skeletal muscle with volumetric muscle loss injury following the transplantation of muscle-ECM. *Biomaterials* 2013;34:3324–3335.
28. Wu X, Corona BT, Chen X, Walters TJ. A standardized rat model of volumetric muscle loss injury for the development of tissue engineering therapies. *Biores Open Access* 2012;1:280–290.
29. Corona BT, Balog EM, Doyle JA, Rupp JC, Luke RC, Ingalls CP. Juncophilin damage contributes to early strength deficits and EC coupling failure after eccentric contractions. *Am J Physiol Cell Physiol* 2010;298:C365–376.
30. Wang R, Urso ML, Zambraski EJ, Rader EP, Campbell KP, Liang BT. Adenosine A receptor stimulation induces protection of skeletal muscle from eccentric exercise-mediated injury. *Am J Physiol Regul Integr Comp Physiol* 2010;299:R259–267.
31. Cai C, Masumiya H, Weisleder N, Matsuda N, Nishi M, Hwang M, *et al.* MG53 nucleates assembly of cell membrane repair machinery. *Nat Cell Biol* 2009;11:56–64.
32. Corona BT, Garg K, Roe JL, Zhu H, Park KH, Ma J, *et al.* Effect of recombinant human MG53 protein on tourniquet-induced ischemia-reperfusion injury in rat muscle. *Muscle Nerve* 2014;49:919–921.
33. Meyer GA, Lieber RL. Skeletal muscle fibrosis develops in response to desmin deletion. *Am J Physiol Cell Physiol* 2012;302:C1609–1620.
34. Corona BT, Machingal MA, Criswell T, Vadhavkar M, Dannahower AC, Bergman C, *et al.* Further development of a tissue engineered muscle repair construct in vitro for enhanced functional recovery following implantation in vivo in a murine model of volumetric muscle loss injury. *Tissue Eng A* 2012;18:1213–1228.
35. Criswell TL, Corona BT, Ward CL, Miller M, Patel M, Wang Z, *et al.* Compression-induced muscle injury in rats that mimics compartment syndrome in humans. *Am J Pathol* 2012;180:787–797.
36. Rantanen J, Ranne J, Hurme T, Kalimo H. Denervated segments of injured skeletal muscle fibers are reinnervated by newly formed neuromuscular junctions. *J Neuropathol Exp Neurol* 1995;54:188–194.
37. Vaitinen S, Hurme T, Rantanen J, Kalimo H. Transected myofibers may remain permanently divided in two parts. *Neuromuscul Disord* 2002;12:584–587.
38. Warren GL, Ingalls CP, Lowe DA, Armstrong RB. What mechanisms contribute to the strength loss that occurs during and in the recovery from skeletal muscle injury? *J Orthop Sports Phys Ther* 2002;32:58–64.
39. Lowe DA, Warren GL, Ingalls CP, Boorstein DB, Armstrong RB. Muscle function and protein metabolism after initiation of eccentric contraction-induced injury. *J Appl Physiol* 1995;79:1260–1270.
40. Lehti TM, Kalliokoski R, Komulainen J. Repeated bout effect on the cytoskeletal proteins titin, desmin, and dystrophin in rat skeletal muscle. *J Muscle Res Cell Motil* 2007;28:39–47.
41. Ingalls CP, Warren GL, Armstrong RB. Dissociation of force production from MHC and actin contents in muscles injured by eccentric contractions. *J Muscle Res Cell Motil* 1998;19:215–224.
42. Ingalls CP, Warren GL, Williams JH, Ward CW, Armstrong RB. E-C coupling failure in mouse EDL muscle after in vivo eccentric contractions. *J Appl Physiol* 1998;85:58–67.
43. Balnave CD, Allen DG. Intracellular calcium and force in single mouse muscle fibres following repeated contractions with stretch. *J Physiol* 1995;488:25–36.
44. Hirata Y, Brotto M, Weisleder N, Chu Y, Lin P, Zhao X, *et al.* Uncoupling store-operated Ca^{2+} entry and altered Ca^{2+} release from sarcoplasmic reticulum through silencing of juncophilin genes. *Biophys J* 2006;90:4418–4427.
45. Murphy RM, Dutka TL, Horvath D, Bell JR, Delbridge LM, Lamb GD. Ca^{2+} -dependent proteolysis of juncophilin-1 and juncophilin-2 in skeletal and cardiac muscle. *J Physiol* 2013;591:719–729.
46. Walters TJ, Kragh JF, Baer DG. Influence of fiber-type composition on recovery from tourniquet-induced skeletal muscle ischemia-reperfusion injury. *Appl Physiol Nutr Metab* 2008;33:272–281.
47. Blaisdell FW. The pathophysiology of skeletal muscle ischemia and the reperfusion syndrome: a review. *Cardiovasc Surg* 2002;10:620–630.
48. Korthals JK, Maki T, Gieron MA. Nerve and muscle vulnerability to ischemia. *J Neurol Sci* 1985;71:283–290.
49. Fox JL, Kenmore PI. The effect of ischemia on nerve conduction. *Exp Neurol* 1967;17:403–419.
50. Chervu A, Moore WS, Homsher E, Quinones-Baldrich WJ. Differential recovery of skeletal muscle and peripheral nerve function after ischemia and reperfusion. *J Surg Res* 1989;47:12–19.
51. Ingalls CP, Wenke JC, Nofal T, Armstrong RB. Adaptation to lengthening contraction-induced injury in mouse muscle. *J Appl Physiol* 2004;97:1067–1076.
52. Armstrong RB, Phelps RO. Muscle fiber type composition of the rat hindlimb. *Am J Anat* 1984;171:259–272.
53. Petersen AM, Pedersen BK. The anti-inflammatory effect of exercise. *J Appl Physiol* 2005;98:1154–1162.
54. Best TM, Hunter KD. Muscle injury and repair. *Phys Med Rehabil Clin N Am* 2000;11:251–266.
55. Jarvinen M. Healing of a crush injury in rat striated muscle. 2. A histological study of the effect of early mobilization and immobilization on the repair processes. *Acta Pathol Microbiol Scand Pathol* 1975;83:269–282.
56. Jarvinen M. Healing of a crush injury in rat striated muscle. 4. Effect of early mobilization and immobilization on the tensile properties of gastrocnemius muscle. *Acta Chir Scand* 1976;142:47–56.
57. Jarvinen M. Healing of a crush injury in rat striated muscle. 3. A micro-angiographical study of the effect of early mobilization and immobilization on capillary ingrowth. *Acta Pathol Microbiol Scand Pathol* 1976;84:85–94.
58. Kvist H, Jarvinen M, Sorvari T. Effect of mobilization and immobilization on the healing of contusion injury in muscle. A preliminary report of a histological study in rats. *Scand J Rehabil Med* 1974;6:134–140.
59. Lehto MU, Jarvinen MJ. Muscle injuries, their healing process and treatment. *Ann Chir Gynaecol* 1991;80:102–108.
60. Kaariainen M, Liljamo T, Peltto-Huikko M, Heino J, Jarvinen M, Kalimo H. Regulation of alpha7 integrin by mechanical stress during skeletal muscle regeneration. *Neuromuscul Disord* 2001;11:360–369.
61. Kannus P, Jozsa L, Jarvinen TL, Kvist M, Vieno T, Jarvinen TA, *et al.* Free mobilization and low- to high-intensity exercise in immobilization-induced muscle atrophy. *J Appl Physiol* 1998;84:1418–1424.
62. Kannus P, Jozsa L, Kvist M, Jarvinen T, Jarvinen M. Effects of immobilization and subsequent low- and high-intensity exercise on morphology of rat calf muscles. *Scand J Med Sci Sports* 1998;8:160–171.

Supplemental Figure 1. Voluntary wheel running activity effect on indices of mitochondrial content and biogenesis. A) Protein levels of cytochrome C and B) gene expression of PGC1alpha were measured at 4 days post injury in control (uninjured) and injured TA muscles from sedentary and active rats. * $\neq$ Active control. Note that active control PGC-alpha approached a ~2-fold upregulation compared to sedentary control muscle.

Supplemental Figure 2. Histological

analysis of regenerating fibers 28 days post-injury. TA muscle cross-sections stained with H&E underwent semi-quantitative analysis for fibers with centrally located nuclei. The percentage of fibers with “normal” or centrally located nuclei were calculated (> 5,000

fibers were analyzed per group). Scale bars = 100 μm . No significant differences were observed.

Supplemental Figure 3. Fiber cross-sectional area in TA muscle from active and sedentary rats 28 days post-injury. TA muscle cross-sections from A) sedentary and B) active rats were probed for laminin (green) and nuclei (DAPI; blue). C) Mean fiber cross-sectional area was determined using an automated macro in Image J. * Sedentary $\neq$ Active, $P < 0.05$. D, E) Frequency distribution of muscle fiber cross-sectional area in TA muscles from sedentary and active rats, respectively ($> 4,000$ fibers per group were analyzed).