Activity Attenuates Skeletal Muscle Fiber Damage After Ischemia And Reperfusion

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EXTREMITY INJURIES ARE HIGHLY PREVALENT IN BOTH MILITARY AND CIVILIAN TRAUMA, AND THEY OFTEN INVOLVE SKELETAL MUSCLE. 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. 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]. THE UNDERLYING MOLECULAR MECHANISMS OF I/R INJURY ARE FAIRLY WELL UNDERSTOOD, AND A NUMBER OF PROTECTIVE AGENTS 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. 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. Cardioprotective factors include increased collateral circulation, induction of heat shock proteins, and improved antioxidant capacity. Interestingly, these factors are also increased in skeletal muscle in response to aerobic activity, 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. Clinically, rehabilitation focuses primarily on muscle strengthening and restoring range of motion through stretching. Treadmill running has been found to be essential for optimal structural organization after muscle contusion. 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, 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
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. 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 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 hallicus longus) was done repeatedly at 28 and 2 days preinjury and 4, 14, and 28 days postinjury, using a dual-mode servomotor (Model S88; Aurora Scientific, Inc.) and methodology we described previously for mice and rats. 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, 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 μl/g body weight). Tibialis anterior (TA) muscles were then frozen and sectioned for histological analysis as described previously. 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.

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 Lieber and by our group. 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 μm² and a circularity between 0.3 and 1.0 were included for analysis.
to remove the measurement of neurovascular bundles, optically fused fibers, and oblique fibers. 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 \((n = 5 \text{ muscles per group})\), as described previously. 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 transcribed 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 (ACT) value. Expression levels for each mRNA transcript \((n = 4 \text{ per group})\) were determined by the 2^−ΔΔCT method by normalizing each group to the uninjured muscle of the sedentary group. 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 \(z = 0.05\). 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

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Values expressed as mean ± standard error of the mean.
*Significantly different from sedentary \((P < 0.05)\).
†Significantly different from uninjured \((P < 0.05)\).
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 by 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 ± 3.8% of TA muscle cross-sectional area in active rats compared with 36.5 ± 3.3% in sedentary rats ($P < 0.001$; $n = 5$ or 6 per group) (Fig. 2C and D). There was also a notably greater 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 ~270-fold 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-$eta$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. 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 ~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 (~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 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 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 coupling 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, which is susceptible to mechanical and oxidative stress, 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, 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 1α (PGC1α) expression, which is associated with mitochondrial biogenesis, was also measured in TA muscles 4 days postinjury (n = 4 per group). In all injured muscles, PGC1α 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 rats.
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 ± 2.24%; $P = 0.02$). By 28 days postinjury, active rats had reached preinjury values, whereas sedentary rats still exhibited an 18.2 ± 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**

Anecdotally, 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. Injurious skeletal muscle fibers after I/R is likely to manifest strength deficits via the loss of force-bearing proteins and disruption of voltage-induced $\text{Ca}^{2+}$ release from the sarcoplasmic reticulum (SR). In addition, muscle fiber lesions can also create temporary denervated abjunctural fiber segments. 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 ~30% of the strength deficit was due strictly to disruption of neural activation of functional muscle fibers to include abjunctural fiber segments, however, the nature of neural disruption was not specifically investigated. The remaining ~70% of the observed neural-evoked torque deficit was attributed to...
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$^{2+}$ release) in the remaining intact muscle fibers. Furthermore, junctophilin 1 protein content was reduced by ~50% in the injured muscles from sedentary and active rats (Fig. 4). Junctophilin 1 is critical to voltage-induced Ca$^{2+}$ release and has been shown to be damaged in response to mechanical and Ca$^{2+}$-dependent proteolysis secondary to oxidative stress. These findings are consistent with the idea that EC coupling is a positive response to muscle injury 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. The sensitivity of skeletal muscle to I/R is a function of fiber type composition; that is, FOG fibers are less sensitive than FG. 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 (PGC1α), 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. The activity-induced enhanced anti-inflammatory response may have played a role in mitigating I/R-induced muscle damage.

![Diagram](image-url)
Although greater activity may be needed to protect healing. These findings broadly highlight that, the initial injury rather than an accelerated rate of recovery. Therefore, the greater recovery of strength after 28 days was essentially the same, at 24%–28%.

Actual rate of strength recovery between 14 and 28 days was consistent with significantly greater strength at both 14 and 28 days in active 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. 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

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. * ≠ Active control. Note that active control PGC-alpha approached a ~2-fold upregulation compared to sedentary control muscle.
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 from laminin (green) and nuclei (DAPI; blue). C) Mean fiber cross-sectional area was determined using an automated macro in Image J. * Sedentary ≠ 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).