RESEARCH ARTICLE

Voluntary running protects against neuromuscular dysfunction following hindlimb ischemia-reperfusion in mice

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Wilson RJ, Drake JC, Cui D, Ritger ML, Guan Y, Call JA, Zhang M, Leitner LM, Gödecke A, Yan Z. Voluntary running protects against neuromuscular dysfunction following hindlimb ischemia-reperfusion in mice. *J Appl Physiol* 126: 193–201, 2019. First published November 15, 2018; doi:10.1152/japplphysiol.00358.2018.—Ischemia-reperfusion (IR) due to temporary restriction of blood flow causes tissue/organ damages under various disease conditions, including stroke, myocardial infarction, trauma, and orthopedic surgery. In the limbs, IR injury to motor nerves and muscle fibers causes reduced mobility and quality of life. Endurance exercise training has been shown to increase tissue resistance to numerous pathological insults. To elucidate the impact of endurance exercise training on IR injury in skeletal muscle, sedentary and exercise-trained mice (5 wk of voluntary running) were subjected to ischemia by unilateral application of a rubber band tourniquet above the femur for 1 h, followed by reperfusion. IR caused significant muscle injury and denervation at neuromuscular junction (NMJ) as early as 3 h after tourniquet release as well as depressed muscle strength and neuromuscular transmission in sedentary mice. Despite similar degrees of muscle atrophy and oxidative stress, exercise-trained mice had significantly reduced muscle injury and denervation at NMJ with improved regeneration and functional recovery following IR. Together, these data suggest that endurance exercise training preserves motor nerve and myofiber structure and function from IR injury and promote functional regeneration.

NEW & NOTEWORTHY This work provides the first evidence that preemptive voluntary wheel running reduces neuromuscular dysfunction following ischemia-reperfusion injury in skeletal muscle. These findings may alter clinical practices in which a tourniquet is used to modulate blood flow.

endurance exercise training; ischemia reperfusion; mitochondria; motor nerve; neuromuscular junction; oxidative stress; skeletal muscle

INTRODUCTION

Ischemia-reperfusion (IR) injury due to reestablishment of blood flow after a temporary lapse is common to many debilitating diseases and a corollary to some clinical procedures. Skeletal muscle as an organ is particularly relevant since, as a common practice in certain types of surgery or as a first response to traumatic injury, a tourniquet is often used to prohibit hemorrhage and exsanguination or provide a bloodless operating field (4, 38). The negative consequences of this procedure include muscle weakness, atrophy, and temporary or permanent nerve damage, all of which hinder the functional recovery (11, 18, 37, 52, 54). For example, ~26% of patients recovering from total knee arthroplasty in which a tourniquet was used reported complications, including profound limb swelling, numbness, and weakness (52, 53). Severe cases will require amputation. As recently as 2008, it was reported that among ~140 million patients with peripheral arterial disease who suffer an acute ischemic event, ~10–30% required amputation within 30 days (21). Thus, limb IR injury poses a significant clinical problem, and despite its prevalence, there is no reliable intervention (51, 64, 67, 75).

The compound cellular alterations accrued during ischemia and reperfusion determine the extent of pathology. This includes intracellular ion imbalance (27), destabilization of the plasma membrane (81), and accumulation of metabolic intermediates (16) during ischemia as well as excessive generation of reactive oxygen species (ROS), plasma membrane rupture (30), activation of inflammatory cascades (12, 79), and necroptosis (46) during reperfusion. Given the diversity of deleterious pathways activated by IR, the best intervention(s) is likely to be the one that could assuage multiple pathologies rather than one component. Indeed, remote preconditioning, which involves repeated short bouts of ischemia in an organ/tissue other than the target organ/tissue before the prolonged ischemic event, has been found to attenuate IR injury in experimental models in a multifaceted manner (1, 13). However, the efficacy of direct preconditioning of himdlimb is far from optimal for full functional protection, and the ideal timing and duration of preconditioning events are yet to be elucidated (17, 23, 68). Thus, it is of the utmost importance to develop alternative therapeutics that target multiple components of IR injury,

which may allow compound therapies in the future to achieve maximal protection.

Exposure to repeated, low-grade stress provokes adaptations that enhance cellular resistance to future and/or more potent insults, a phenomenon called hormesis (35, 47, 62). In line with this biological phenomenon, endurance exercise training involves transient energetic, oxidative, and mechanical stresses that elicit favorable adaptations both locally and systemically (3, 19, 62). Indeed, endurance exercise training has been shown to lessen IR injury in the heart (7–9, 20, 26, 60), liver (69), and lungs (19), whereas the underlying mechanisms may vary and include enhancement of antioxidant (34, 65, 72, 76, 80) and repair enzyme activity and expression (25, 40, 45, 66), increased Ca^{2+} buffering capacity (43, 63, 82), and improved mitochondrial quality (22, 42, 82). However, there have not been studies investigating the impact of endurance exercise training on the susceptibility of the adapted skeletal muscle to IR injury. If endurance exercise training promotes skeletal muscle resistance to IR, the next question would be whether the protection occurs during the ischemia or reperfusion phase or as a continuation between the two. Additionally, it is not known whether exercise-mediated protection is predominantly motor nerve fibers or myofibers. In the present study, we tested the hypothesis that endurance exercise training is sufficient to protect motor nerve fibers, the neuromuscular junction (NMJ), and/or myofibers against IR injury through a mechanism by reducing oxidative stress. The findings would significantly improve our understanding of the utility and underlying mechanism(s) of endurance exercise training as a therapeutic intervention to attenuate/prevent IR injury.

MATERIALS AND METHODS

Animals. All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Virginia. Male mice were housed in temperature-controlled (21°C) cages in a pathogen-free room with a 12:12-h light-dark cycle and free access to water and normal chow. Inducible whole body MitoTimer transgenic mice (*CAG-CAT-MitoTimer*) were generated as previously described (78). To induce MitoTimer expression, tamoxifen (40 mg/kg ip) was administered daily for 7 days in *CAG-CAT-MitoTimer* mice of 9–12 wk of age, followed by 3 days of recovery before the experimental procedures.

Voluntary running. Voluntary running was set as described previously (40). Briefly, mice in the exercise group were housed individually in cages equipped with running wheels for 5 wk, and sedentary mice were housed in cages not equipped with running wheels. Daily running was recorded via a computerized monitoring system. Running wheels were locked for 24 h before the subsequent experimental procedures to minimize the effect of acute exercise.

Hindlimb ischemia-reperfusion. Hindlimb IR injury was induced as previously described with minor modifications (5, 77). Briefly, under anesthesia (isofluorane in oxygen), a 4.0-oz, 1.8-in. orthodontic rubber band (11-102-03; DENTSPLY GAC International) was applied above the greater tronchanter of the femur using a McGivney Hemorrhodial Ligator to block the blood flow. Mice were conscious and monitored during the 1-h ischemic period before the tourniquet was removed to induce reperfusion.

Creatine kinase activity. Serum creatine kinase activity was measured by using a commercially available kit, following the manufacturer's instructions (MAK116; Sigma Aldrich). For sample preparation, blood was collected from the tail vein before and 3 h after IR, incubated at room temperature for 30 min, and then spun at 1,500 *g* at 4°C for 30 min. The supernatant was saved (serum), aliquoted, and stored at −80°C until further analysis.

In vivo muscle function. Maximal isometric torque of the plantar flexor muscles was assessed as previously described (10, 77) before and 24 h, 72 h, and 7 days after IR injury. Briefly, mice were placed on a heated stage in the supine position under anesthesia (1% isofluorane in oxygen), and the right foot was secured to a foot plate that was attached to a servomotor at 90° relative to the immobilized knee (Model 300C-LR; Aurora Scientific). For nerve-stimulated contractions (Nerve Stim), a pair of Teflon-coated electrodes were inserted percutaneously on both sides of the sciatic nerve ~1 cm proximal to the knee joint. For direct muscle stimulation, electrodes were inserted into the proximal and distal ends of the gastrocnemius muscle. Peak isometric torque (mN/m), which is referred to as strength, was achieved by varying the current delivered to the nerve or muscle and keeping the following parameters constant: 10 V of electric potential, 200-Hz stimulation frequency, 300-ms stimulation duration, and 0.3-ms pulse duration. The force-frequency relationship was determined by incrementally increasing stimulation frequency, with a 45-s resting period between two contractions (10, 20, 30, 40, 60, 80, 100, 125, and 150 Hz). To account for differences in body size among mice during longitudinal studies, torque was normalized by body mass (g), which did not change over the experimental time period. Specific torque was calculated by dividing absolute torque by plantarflexor muscle (gastrocnemius, plantaris, and soleus) wet weight (mg).

Morphological and immunohistological analysis. Morphological and immunohistological analyses of plantaris muscle were performed as previously described (49, 77). Transverse muscle sections (5 μm) were stained with hematoxylin and eosin (H&E) (49) or immunostained using primary and fluorophore-conjugated secondary antibodies. Primary antibodies against both Ncam (ab9018; Abcam) and laminin (MAB1928; Chemicon) were diluted 1:100. Percentage of centralized nuclei and myofibers positive for Ncam in the cytosol were calculated by dividing the number of fibers with aforementioned markers (counted by a blinded investigator) divided by the total number of fibers in three random fields of view per muscle.

MitoTimer analysis. MitoTimer is a mitochondria targeted reporter gene that serves as a sensor of mitochondrial oxidative stress. When MitoTimer is oxidized, it shifts emission wavelength from green fluorescent protein (GFP; excitation/emission 488/518 nm) to Discosoma sp. red protein (DsRed excitation/emission 543/572 nm). Ratiometric analysis of MitoTimer (red/green ratio) is a quantifiable metric of mitochondrial oxidative stress (40, 42, 55, 78). Imaging of MitoTimer in plantaris muscle and sciatic nerve using Olympus Fluoview FV1000 was conducted as previously described (40, 42, 77, 78). Fluorescent intensity of MitoTimer red and green fluorescence was quantified using a custom MatLab-based algorithm from which MitoTimer red/green ratio was calculated. Identical acquisition parameters were used for every sample of the same tissue type.

NMJ analysis. NMJ morphology and occupancy were assessed as previously described (58, 59, 77). Immediately upon harvest, plantaris muscles were fixed in 4% paraformaldehyde for 20 min, washed three times in PBS, blocked in 5% normal goat serum, and incubated with primary antibodies against tubulin β-III (Tuj1, 801201; Covance) 1:100 and synaptic vesicle 2 (SV2, ab32942; Abcam) at 4°C overnight. The muscles were then washed with PBS and incubated with fluorescently conjugated secondary antibodies and Alexa 647-conjugated α-bungarotoxin (B35450; Thermo Scientific) diluted 1:100 in PBS for 30 min (31, 77). Images were acquired using Olympus Fluoview FV1000. To assess all the NMJs, Z-stacks were acquired using both ×20 and ×60 objectives. Only NMJs complete en face acquired at ×60 were analyzed as previously described (59, 77). Maximum intensity Z-stacks were reconstructed in ImageJ (National Institutes of Health) and underwent the following corrections in the order listed: background subtraction (50.0 pixels), despeckling, application of a Gaussian blur (2.0 radius), and conversion to binary. Occupancy was determined by dividing the area of the presynaptic structures by the area of postsynaptic structures (pre- μm²/post-μm² × 100). Denervation is defined as the percentage of total NMJs

in which the occupancy is <5%. A minimum of 30 NMJs were analyzed per muscle.

Immunoblotting. Immediately after harvesting, proteins were extracted from tissues, and immunoblotting was performed as previously described (40, 71). Briefly, tissues were homogenized in 2× sample Laemmli sample buffer containing protease and phosphatase inhibitors (1:10 g tissue/μl buffer), boiled at 95°C for 5 min, and spun at maximum speed for 5 min. The supernatant was transferred to a clean tube, and protein concentration was determined using RC DC assay (Bio-Rad). Equal amounts of protein were separated using SDS-PAGE electrophoresis. Proteins were transferred to nitrocellulose membrane and then blocked with 5% milk in TBST. Membranes were incubated with the following primary antibodies: superoxide dismutase (SOD)1 (ab16831; Abcam), SOD2 (ab13534; Abcam), SOD3 (07-704; Upstate), catalase (ab15834; Abcam), 4-hydroxynonenal (48506; Abcam), and actin (A2066; Sigma-Aldrich).

Statistical analysis. Statistical analyses were performed using GraphPad Prism software, and values are presented as means ± SD. Two-tailed t-test was used for comparisons between sedentary and exercise-trained mice. One-way analysis of variance (ANOVA) was used for comparisons among sham, sedentary, and exercise-trained mice. Two-way ANOVA was used to compare torque produced between sedentary and exercise-trained groups pre- and postinjury. A significant interaction of 0.05 was required to perform a between-variable post hoc analysis, in which case Tukey's honestly significant difference test was performed. $P < 0.05$ is considered statistically significant for all of the analyses described above.

RESULTS

Long-term voluntary running preserves muscle contractile function following IR. To ascertain whether endurance exercise training leads to protection against IR injury in skeletal muscle, we subjected sedentary and exercise-trained mice (following 5 wk of voluntary running) to IR injury with sham-operated mice serving as controls. Myofiber and motor nerve fiber functions were assessed based on total strength of plantar flexor muscles following direct muscle or sciatic nerve stimulation, respectively. These approaches provide insight into muscle contractile function and neuromuscular transmission indicative of myofiber and motor nerve function, respectively (23).

Prior to the injury, body weight (27.4 ± 1.4 g in sedentary mice and 27.0 ± 1.18 g in exercise-trained mice), serum creatine kinase (378 ± 54.5 U/l in sedentary mice and 328 ± 77 U/l in exercise-trained mice), and muscle strength by direct muscle (Fig. 1*A*) and nerve stimulation (Fig. 1*B*) were indistinguishable between sedentary and exercise-trained mice. Exercise-trained mice had significantly greater strength, as shown by greater torque by either direct muscle (Fig. 1*A*) or nerve (Fig. 1*B*) stimulation at 24 h, 72 h, and 7 days following IR. At 7 days, gastrocnemius muscle mass from sedentary and exercise-trained mice was reduced by 22 and 29% ($P > 0.05$ between these two groups), respectively, compared with the sham control mice, suggesting an equal level of myofiber atrophy (Fig. 1*C*). Next, we evaluated the torque-frequency relationship at 7 days. Interestingly, we observed a left shift in the torque-frequency relationship after IR injury, in which 50% of maximal strength of injured muscles was reached at a lower frequency (~30 Hz) than the sham control (~60 Hz). This suggests that either surviving fibers are predominantly slow-twitch fibers or that there was altered Ca^{2+} handling following IR injury. However, we found that exercise-trained mice had greater strength than sedentary mice at submaximal frequencies by direct muscle (Fig. 1*D*) and nerve (Fig. 1*E* stimulation. Together, these findings suggest that exercise training preserves both myofiber and motor nerve function.

Long-term voluntary running does not prevent IR-induced oxidative stress. Oxidative stress and consequent damage to cellular components is a hallmark of IR injury. Reduction in the production of oxidants or enhanced detoxification of oxidants has been found to reduce IR injury across a number of tissues (39, 48). Endurance exercise training has been reported to promote antioxidant defense systems in skeletal muscle (29, 34), which might lead to increased resistance to IR injury. Indeed, we found that expression of superoxide dismutase isoforms 1, 2, and 3 as well as catalase was significantly increased following 5 wk of voluntary running in skeletal muscle (Fig. 2*A*), but not in sciatic nerve (Fig. 2*B*), prompting us to hypothesize that exercise training-mediated protection
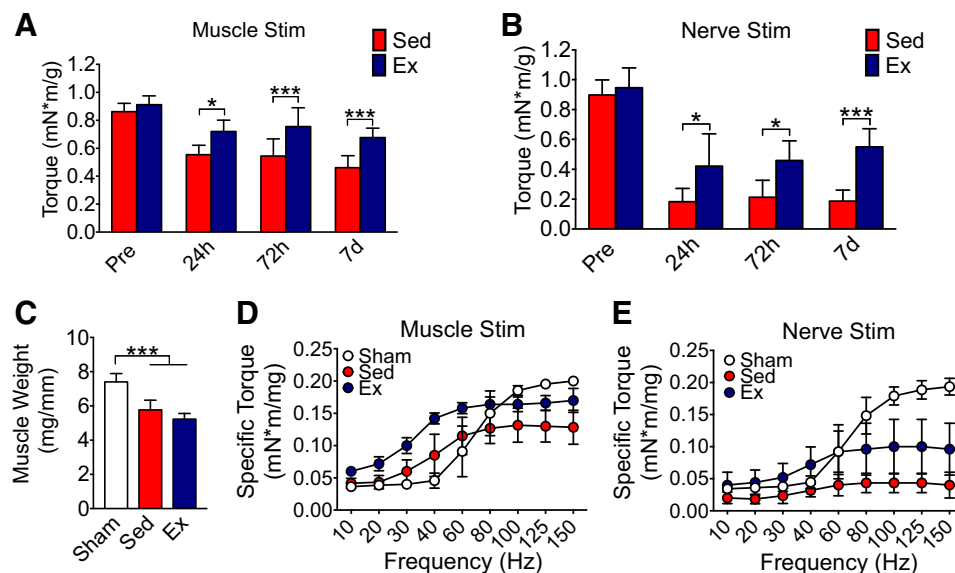

Fig. 1. Long-term voluntary running preserves neuromuscular function following ischemia-reperfusion (IR). To determine whether endurance exercise training provides protection against IR injury-mediated loss of neuromuscular function, sedentary (Sed) and exercise-trained (Ex) mice were subjected to IR, followed by measurements of muscle weight and muscle and nerve function 7 days after IR. *A*: peak isometric torque elicited by direct muscle stimulation before and during recovery from IR, and only statistical differences between Sed and Ex are indicated. *B*: peak isometric torque of plantar flexors elicited by nerve stimulation before and during recovery from IR. For simplicity, only statistical differences between sedentary and exercise are indicated. *C*: gastrocnemius muscle wet weight (mg) normalized to tibia length (mm) to account for differences in body size. *D*: torque-frequency relationship of muscle contractions elicited by direct muscle stimulation 7 days following IR. *E*: force-frequency relationship of muscle contractions elicited by nerve stimulation 7 days following IR ($n = 6$). Data are represented as means ± SD. *$P < 0.05$ and ***$P < 0.001$; $n = 6$.
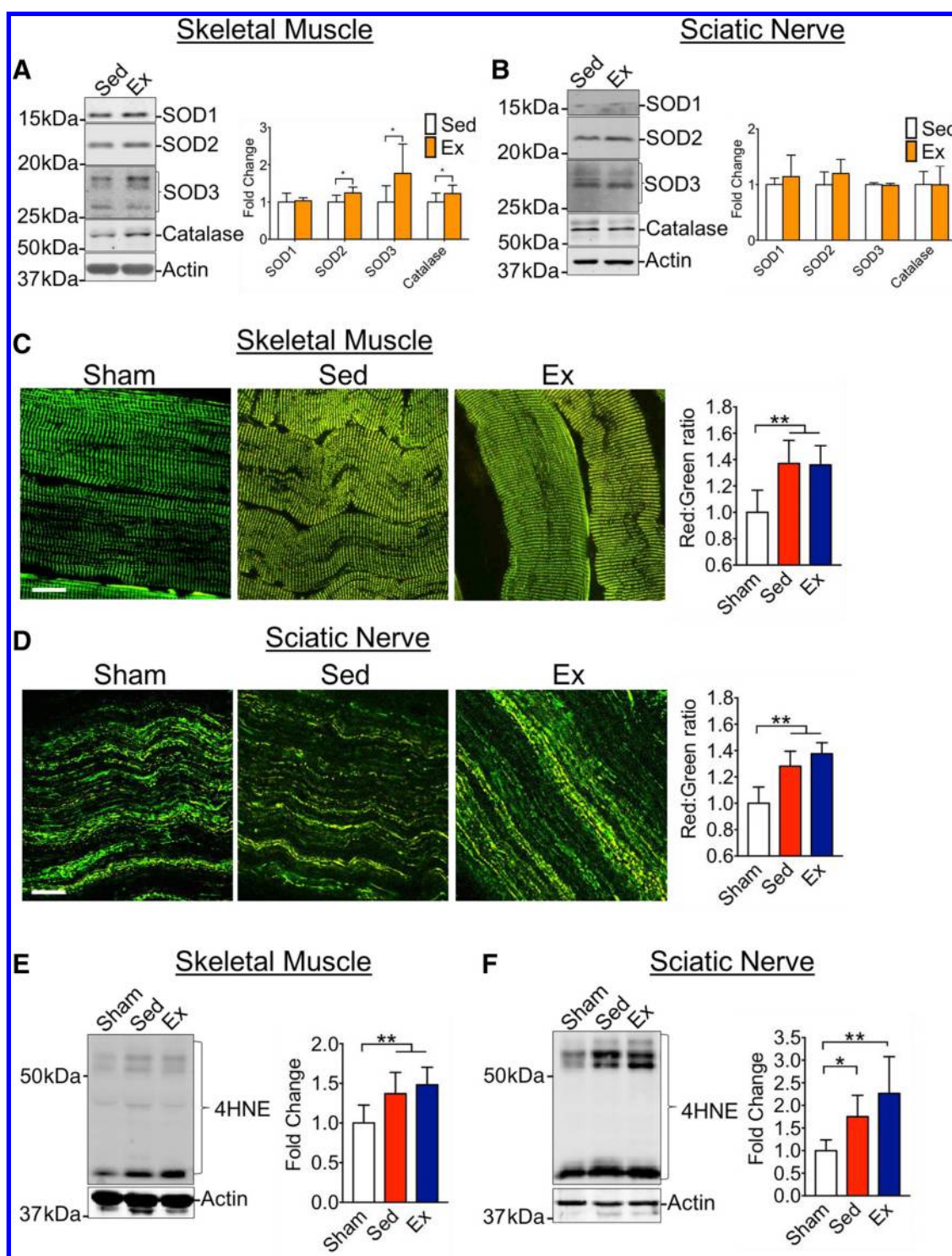
Fig. 2. Long-term voluntary running does not attenuate ischemia-reperfusion (IR)-induced oxidative stress in myofibers and motor nerve. We measured mitochondrial and whole cell markers of oxidative stress in sedentary (Sed) and exercise-trained (Ex) mice following IR. *A*: representative immunoblots and quantification of expression of antioxidant proteins superoxide dismutase (SOD)1, SOD2, and SOD3 and catalase normalized by actin in skeletal muscle ($n = 5$). *B*: representative immunoblot images and quantification of expression of antioxidant proteins SOD1, SOD2, and SOD3 and catalase normalized by actin in sciatic nerve ($n = 5$). *C*: representative confocal images and quantification of MitoTimer red:green ratio in skeletal muscle 3 h after IR. Scale, 25 μm ($n = 4$–7). *D*: representative confocal images and quantification of MitoTimer red/green ratio in sciatic nerve 3 h after IR. Scale, 25 μm ($n = 4$–7). *E*: representative immunoblot images and quantification of 4-hydroxynoneal (4-HNE) in skeletal muscle ($n = 6$). *F*: representative immunoblot images and quantification of 4-HNE in sciatic nerve ($n = 6$). Data are represented as means ± SD. *$P < 0.05$; **$P < 0.01$.

against IR injury was through at least a reduction in oxidative stress in myofibers.

To test this hypothesis, we first evaluated mitochondrial oxidative stress in vivo by using a novel transgenic mouse model with a globally induced expression of the mitochondria reporter gene *MitoTimer* (MitoTimer-Tg). *MitoTimer* encodes a mitochondrial targeted green fluorescent protein that irreversibly switches to Discosoma sp. red fluorescent protein upon oxidation (42, 73). Computer-assisted ratiometric analysis of the MitoTimer red/green fluorescence ratio provides a quantifiable measure of mitochondrial oxidative stress (40, 42, 55, 78). We subjected sedentary and exercise-trained MitoTimer-Tg mice to IR and collected tissues at 3 h. MitoTimer red/green ratio in myofibers (Fig. 2C) and motor nerve exons (Fig. 2D) was indistinguishable between sedentary and exercise-trained mice and higher than the sham control mice, indicating that exercise training does not attenuate IR-induced mitochondrial oxidative stress. Next, we measured 4-hydroxynoneal (4-HNE), a stable product of lipid peroxidation (56, 57), in whole cell lysates. Similarly to the findings of MitoTimer, we observed significant increases of 4-HNE in myofibers (Fig. 2E) and motor nerve (Fig. 2f) 3 h after IR in sedentary and exercise-trained mice when compared with the sham control. Together, these data suggest that the main protective effect of endurance exercise training against IR may not be through an enhanced antioxidant defense with reduced oxidative stress.

Long-term voluntary running attenuates myofiber damage following IR. To assess injury to myofibers, we performed morphological analysis on transverse sections of the plantaris muscle by H&E staining. At 3 h post-IR, skeletal muscle from sedentary mice displayed many rounded myofibers with increased interstitial space, indicative of edema and structural disruption, which was absent in exercise-trained mice (Fig. 3A). To further validate the protection by exercise, we measured the activity of creatine kinase (CK) in the serum, a

clinically relevant marker for IR-induced muscle damage (15, 32, 33). Compared with the sham control mice, serum CK activity increased sixfold in sedentary mice, which was attenuated to 3.5-fold in exercise-trained mice (Fig. 3B). Taken together, the reductions in morphological changes and serum CK are indicative of reduced myofiber damage. We then assessed muscle morphology 7 days following IR. Sedentary mice displayed a significant increase in myofibers with centralized myonuclei, a marker for ongoing muscle regeneration, when compared with the sham control (Fig. 3D). Although there was a trend of increased number of myofibers with centralized myonuclei in exercise-trained mice, it was not statistically significant. In summary, morphological and biochemical analysis of markers of myofiber damage suggests that exercise training improves myofiber resistance to IR-induced injury.

Long-term voluntary running preserves innervation at NMJ following IR. Patients with tourniquet usage may have temporary or permanent motor nerve damage, which contributes to postprocedure muscle weakness and delayed functional recovery (44, 52, 70). Neuromuscular junction (NMJ) is a specialized chemical synapse formed between motor nerve and myofiber that serves as the nexus of neuromuscular transmission. Previous studies have revealed that NMJ is vulnerable to IR injury (74); therefore, we asked whether endurance exercise training could preserve NMJ integrity. We quantified the fluorescent overlap of the presynaptic neuron-specific class III β-tubulin (Tuj1) with the postsynaptic acetylcholine receptors (AchR) in plantaris muscle as a parameter of innervation at NMJ. At 3 h after IR, Tuj1 florescence that overlaps with AchR was profoundly decreased compared with the sham control (Fig. 4A). However, significantly less NMJ showed this change in skeletal muscle of exercise-trained mice. To further ascertain long-term impact of IR on innervation, we measured intramuscular expression of neuronal cell adhesion marker (Ncam), a marker of denervation and muscle regeneration (14,
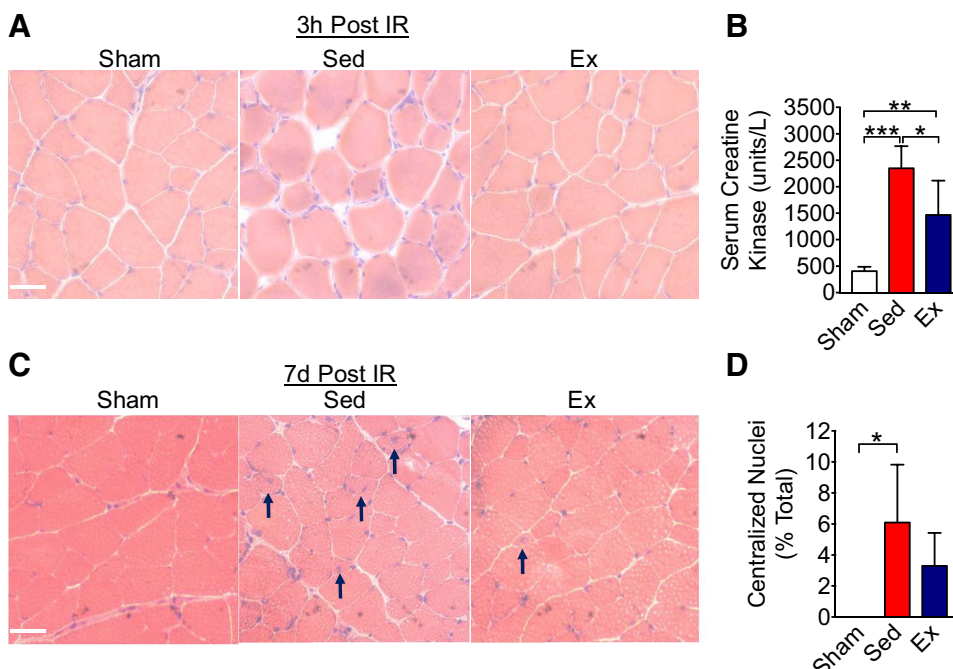

Fig. 3. Long-term voluntary running renders myofibers resistant to ischemia-reperfusion (IR). Morphological and biochemical evaluations of muscle damage were conducted following IR. *A*: representative images of hematoxylin and eosin (H & E)-stained transverse sections of skeletal muscle 3 h after IR. Scale bar, 50 μm. *B*: serum creatine kinase activity 3 h after IR. *C*: representative images of H & E-stained transverse sections of skeletal muscle 7 days after IR. Scale bar, 50 μm. *D*: %total myofibers with centralized nuclei. Data are represented as means ± SD. *$P <$ 0.05, **$P < 0.01$, and ***$P < 0.001$; $n = 6$. Sed, sedentary; Ex, exercise trained.
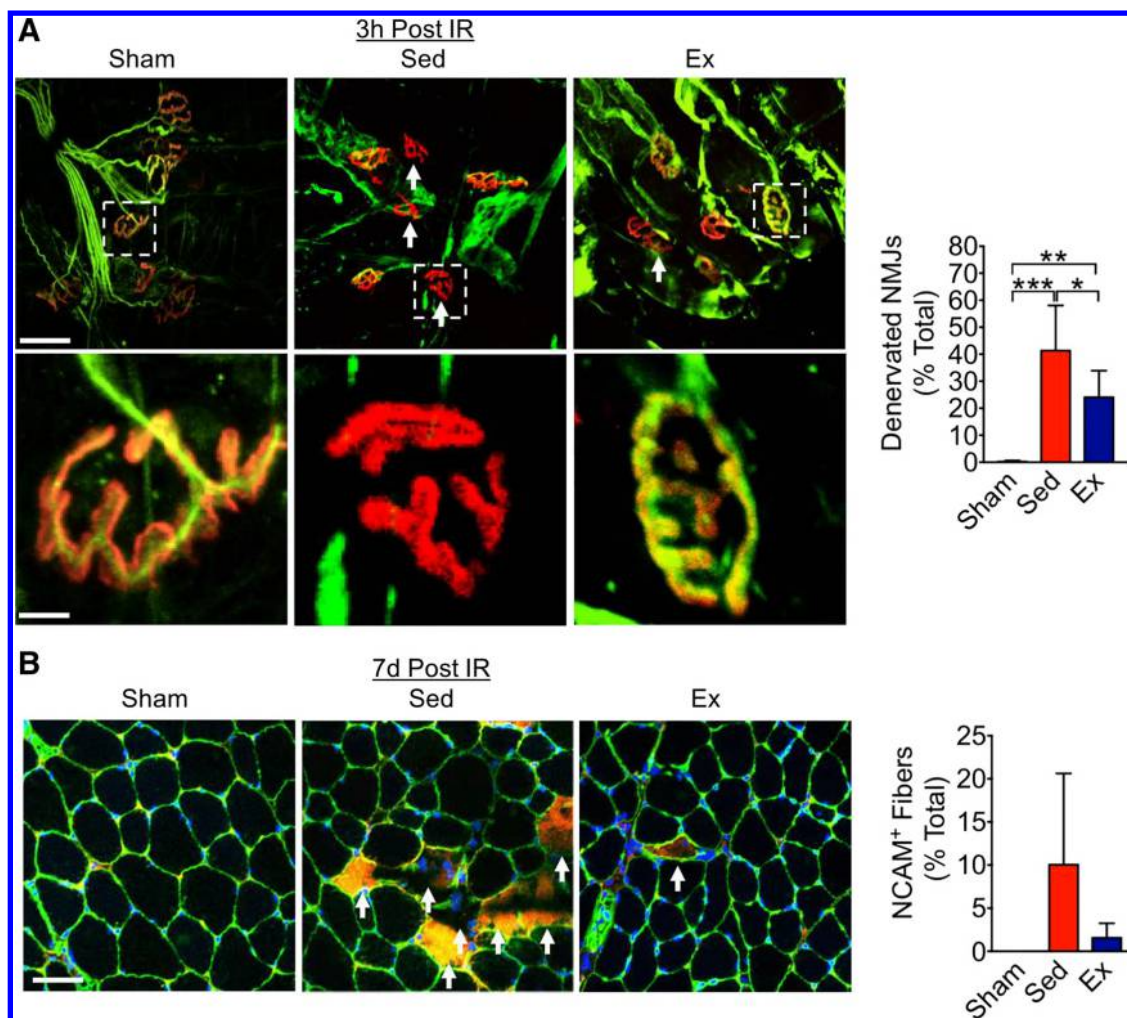
Fig. 4. Long-term voluntary running attenuates denervation of skeletal muscle following ischemia-reperfusion (IR). To elucidate the consequence of endurance exercise training on skeletal muscle innervation, muscles were collected from sedentary (Sed) and exercise-trained (Ex) mice follwing IR, and innervation at neuromuscular junction (NMJ) and muscle denervation were measured using immunofluorescent techniques. *A*: representative confocal images of presynaptic motor neurons identified by Tuj1 (green) and postsynaptic acetylcholine receptors detected with α-bungarotoxin (red) and quantification of denervated NMJs 3 h after injury. Scale bars, 20 (*top*) and 5 μm (*bottom*), respectively (*$P < 0.05$ and ***$P < 0.001$; $n = 8$). *B*: representative confocal images of transverse sections of plantaris muscle expressing cytosolic Ncam (red) and laminin (green) and DAPI staining (blue) and quantification of %cytosolic Ncam$^+$ myofibers 7 days following IR. Mice in the Sed group had a trend of increase toward significance ($P = 0.053$). Scale bar, 100 μm ($n = 5$). Data are represented as means ± SD.

28, 36). On *day 7* following IR, sedentary mice, but not exercise-trained mice, showed a clear trend of increased cytosolic expression of Ncam compared with the sham control ($P = 0.053$; Fig. 4*B*). These data collectively demonstrate that exercise training attenuates denervation at NMJ following IR.

DISCUSSION

Impairment of neuromuscular function is an inherent risk in procedures that employ a tourniquet to block blood flow. The clinical manifestations of IR injury in this context are myofiber atrophy, weakness, limb numbness, and temporary or permanent paralysis, all of which jeopardize the quality of life and amplify the incidence of morbidity and mortality. Although we have recently demonstrated that IR injury to NMJ can be attenuated by targeted enhancement of mitochondrial protein *S*-nitrosation (77), there remains a need to develop an effective and accessible physiological intervention that also protects myofibers. Endurance exercise training is one of the most

feasible candidates in this regard. Endurance exercise has been shown to improve myocardial tolerance to IR injury in a manner that is analogous to preconditioning (8, 61). In fact, during strenuous isotonic contractions, such as those elicited during exercise, arterial blood flow to skeletal muscle is arrested and is restored only when the muscle relaxes, effectively causing brief rounds of IR (2). However, whether endurance exercise training confers such benefits in skeletal muscle remained unaddressed. This study has provided the first evidence that endurance exercise training attenuates IR-induced neuromuscular derangement on the functional, morphological, cellular, and molecular levels.

In this study, we assessed neuromuscular function by measuring and comparing muscle tetanic torque produced via muscle and motor nerve stimulations. Impairments in muscle contraction in response to direct muscle stimulation reveal reduced intrinsic muscle contractile capacity, perhaps as a result of myopathies, including but not limited to abnormalities

in protein degradation/synthesis, cross-bridge cycling, and/or excitation-contraction coupling. We observed clear biochemical evidence of injury as well as concurrent muscle edema and rounding of fibers by IR, which was attenuated in exercise-trained mice. These findings suggest that endurance exercise training substantially reduces IR injury to myofibers. Moreover, the percentage of myofibers with centralized nuclei was significantly increased 7 days after IR in sedentary mice, whereas this increase was not statistically significant in exercise-trained mice. Considering these findings in summary, we conclude that endurance exercise training resulted in fewer damaged myofibers by IR. Alternatively, the same number of myofibers were affected, but to a lesser degree in exercise-trained mice or a mixture of both. Future studies are necessary to determine which phenomena predominate.

Assessment of muscle contraction in response to sciatic nerve stimulation and innervation at NMJ provide insight into motor nerve function. The former assesses neuromuscular transmission whereby nerve impulses initiate muscle contraction, and the latter reveals the structural integrity underlying this important function. We observed a dramatic decrease in nerve-stimulated muscle tetanic torque concurrent with denervation at NMJ, supporting the notion of compromised neuromuscular transmission following IR. This functional parameter was significantly preserved in exercise-trained mice following IR accompanied by attenuated denervation at NMJ. These data suggest that exercise training preserves motor nerve function, at least in part, by preserving innervation at NMJ.

Acute bout(s) of exercise causes transient oxidative stress in skeletal muscle and other remote tissue/organs, which may trigger adaptive responses and ultimately render the affected tissues/organs more resistant to ensuing future stresses (please see reviews in Refs. 6, 22, and 24). A seemingly important adaptation induced by endurance exercise training is increased expression of enzymes in the antioxidant defense system. Consistent with the findings by our and other groups, we found that long-term voluntary running led to modest increases of antioxidant enzymes in myofibers (34, 50). However, IR-induced cytosolic and mitochondrial oxidative stresses assessed by a fluorescent reporter for mitochondrial oxidative stress as well as 4-HNE mitochondrial protein adducts were not attenuated in myofibers of exercise-trained mice. The most straightforward explanation is that endurance exercise training-induced increases in antioxidant enzymes are not sufficient to prevent oxidative stress induced by IR.

We have shown clear evidence of muscle injury and degeneration/regeneration following IR in sedentary mice, as indicated by morphological disruptions and appearance of centralized myonuclei, respectively. Exercise-trained mice had significantly attenuated increases of these parameters, consistent with the notion that myofibers from exercise-trained mice are more resistant to IR injury despite the fact that they endure similar oxidative stress following IR. It is equally intriguing that despite the similar degree of oxidative stress in the motor nerve, exercise-trained mice showed protected neuromuscular transmission and innervation at NMJ. The underlying mechanisms for endurance exercise training-induced resistance to IR injury in motor nerve and skeletal muscle remain a mystery and warrant further investigations.

In conclusion, this study has provided the first evidence that endurance exercise training is sufficient to attenuate IR injury

in motor nerves and myofibers, thus preserving neuromuscular function and promote functional regeneration from IR. This exercise training-induced protection may not be through reduced oxidative stress in the myofibers and motor nerve. Collectively, our findings support a new application of endurance exercise training with strong clinical implications where endurance exercise regime could be prescribed in preparation for surgeries or procedures that will employ a tourniquet. Whether injury and/or recovery could be augmented by exercise training after injury or coupling exercise training with other interventions, such as the aforementioned augmentation of mitochondrial protein *S*-nitrosation, is a compelling question worthy of investigation. Finally, these discoveries provide a foundation for future studies to elucidate the precise mechanism(s) of exercise training-mediated protection against IR injury, which may be relevant to other IR-related injuries or diseases.

DISCLOSURES

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

AUTHOR CONTRIBUTIONS

R.J.W. and Z.Y. conceived and designed research; R.J.W., Y.G., M.Z., and L.M.L. performed experiments; R.J.W. and M.L.R. analyzed data; R.J.W., J.A.C., and Z.Y. interpreted results of experiments; R.J.W. prepared figures; R.J.W. drafted manuscript; R.J.W., J.C.D., D.C., J.A.C., L.M.L., A.G., and Z.Y. edited and revised manuscript; R.J.W., J.C.D., D.C., M.L.R., Y.G., J.A.C., M.Z., L.M.L., A.G., and Z.Y. approved final version of manuscript.

REFERENCES

1. **Ali ZA, Callaghan CJ, Lim E, Ali AA, Nouraei SAR, Akthar AM, Boyle JR, Varty K, Kharbanda RK, Dutka DP, Gaunt ME.** Remote ischemic preconditioning reduces myocardial and renal injury after elective abdominal aortic aneurysm repair: a randomized controlled trial. *Circulation* 116, *Suppl*: I98–I105, 2007. doi:10.1161/circulationaha.106.679167.

2. **Anrep GV, von Saalfeld E.** The blood flow through the skeletal muscle in relation to its contraction. *J Physiol* 85: 375–399, 1935. doi:10.1113/jphysiol.1935.sp003326.

3. **Arsenault BJ, Larose E.** Appreciating the local and systemic effects of exercise training on vascular health. *Atherosclerosis* 231: 15–17, 2013. doi:10.1016/j.atherosclerosis.2013.08.022.

4. **Beekley AC, Sebesta JA, Blackbourne LH, Herbert GS, Kauvar DS, Baer DG, Walters TJ, Mullenix PS, Holcomb JB; 31st Combat Support Hospital Research Group.** Prehospital tourniquet use in Operation Iraqi Freedom: effect on hemorrhage control and outcomes. *J Trauma* 64, *Suppl*: S28–S37, 2008. doi:10.1097/TA.0b013e318160937e.

5. **Bonheur JA, Albadawi H, Patton GM, Watkins MT.** A noninvasive murine model of hind limb ischemia-reperfusion injury. *J Surg Res* 116: 55–63, 2004. doi:10.1016/S0022-4804(03)00232-4.

6. **Booth FW, Thomason DB.** Molecular and cellular adaptation of muscle in response to exercise: perspectives of various models. *Physiol Rev* 71: 541–585, 1991. doi:10.1152/physrev.1991.71.2.541.

7. **Borges JP, de Oliveira França G, Cruz MD, Lanza R, do Nascimento AR, Lessa MA.** Aerobic exercise training induces superior cardioprotection following myocardial ischemia reperfusion injury than a single aerobic exercise session in rats. *Mot Rev Educ Física* 23: 1–5, 2017. doi:10.1590/s1980-6574201700si0011.

8. **Borges JP, Lessa MA.** Mechanisms involved in exercise-induced cardioprotection: a systematic review. *Arq Bras Cardiol* 105: 71–81, 2015. doi:10.5935/abc.20150024.

9. **Brown DA, Lynch JM, Armstrong CJ, Caruso NM, Ehlers LB, Johnson MS, Moore RL.** Susceptibility of the heart to ischaemia-reperfusion injury and exercise-induced cardioprotection are sex-dependent in the rat. *J Physiol* 564: 619–630, 2005. doi:10.1113/jphysiol.2004.081323.

10. **Call JA, Wilson RJ, Laker RC, Zhang M, Kundu M, Yan Z.** Ulk1-mediated autophagy plays an essential role in mitochondrial remodeling and functional regeneration of skeletal muscle. *Am J Physiol Cell Physiol* 312: C724–C732, 2017. doi:10.1152/ajpcell.00348.2016.

11. **Carmo-Araújo EM, Dal-Pai-Silva M, Dal-Pai V, Cecchini R, Anjos Ferreira AL.** Ischaemia and reperfusion effects on skeletal muscle tissue: morphological and histochemical studies. *Int J Exp Pathol* 88: 147–154, 2007. doi:10.1111/j.1365-2613.2007.00526.x.

12. **Chan RK, Ding G, Verna N, Ibrahim S, Oakes S, Austen WG Jr, Hechtman HB, Moore FD Jr.** IgM binding to injured tissue precedes complement activation during skeletal muscle ischemia-reperfusion. *J Surg Res* 122: 29–35, 2004. doi:10.1016/j.jss.2004.07.005.

13. **Cheung MMH, Kharbanda RK, Konstantinov IE, Shimizu M, Frndova H, Li J, Holtby HM, Cox PN, Smallhorn JF, Van Arsdell GS, Redington AN; First Clinical Application in Humans.** Randomized controlled trial of the effects of remote ischemic preconditioning on children undergoing cardiac surgery: first clinical application in humans. *J Am Coll Cardiol* 47: 2277–2282, 2006. doi:10.1016/j.jacc.2006.01.066.

14. **Chipman PH, Schachner M, Rafuse VF.** Presynaptic NCAM is required for motor neurons to functionally expand their peripheral field of innervation in partially denervated muscles. *J Neurosci* 34: 10497–10510, 2014. doi:10.1523/JNEUROSCI.0697-14.2014.

15. **Chiu D, Wang HH, Blumenthal MR.** Creatine phosphokinase release as a measure of tourniquet effect on skeletal muscle. *Arch Surg* 111: 71–74, 1976. doi:10.1001/archsurg.1976.01360190073013.

16. **Chouchani ET, Pell VR, Gaude E, Aksentijević D, Sundier SY, Robb EL, Logan A, Nadtochiy SM, Ord ENJ, Smith AC, Eyassu F, Shirley R, Hu C-H, Dare AJ, James AM, Rogatti S, Hartley RC, Eaton S, Costa ASH, Brookes PS, Davidson SM, Duchen MR, Saeb-Parsy K, Shattock MJ, Robinson AJ, Work LM, Frezza C, Krieg T, Murphy MP.** Ischaemic accumulation of succinate controls reperfusion injury through mitochondrial ROS. *Nature* 515: 431–435, 2014. doi:10.1038/nature13909.

17. **Coban YK, Ciralik H, Kurutas EB.** Ischemic preconditioning reduces the severity of ischemia-reperfusion injury of peripheral nerve in rats. *J Brachial Plex Peripher Nerve Inj* 1: 2, 2006. doi:10.1186/1749-7221-1-2.

18. **Dash RK, Li Y, Kim J, Beard DA, Saidel GM, Cabrera ME.** Metabolic dynamics in skeletal muscle during acute reduction in blood flow and oxygen supply to mitochondria: in-silico studies using a multi-scale, top-down integrated model. *PLoS One* 3: e3168, 2008. doi:10.1371/journal.pone.0003168.

19. **Delbin MA, Antunes E, Zanesco A.** Papel do exercício físico na isquemia/reperfusão pulmonar e resposta inflamatória. *Rev Bras Cir Cardiovasc* 24: 552–561, 2009. doi:10.1590/S0102-76382009000500017.

20. **Demirel HA, Powers SK, Zergeroglu MA, Shanely RA, Hamilton K, Coombes J, Naito H.** Short-term exercise improves myocardial tolerance to in vivo ischemia-reperfusion in the rat. *J Appl Physiol (1985)* 91: 2205–2212, 2001. doi:10.1152/jappl.2001.91.5.2205.

21. **Diehm N, Schillinger M, Minar E, Gretener S, Baumgartner I.** TASC II section E3 on the treatment of acute limb ischemia: commentary from European interventionists. *J Endovasc Ther* 15: 126–128, 2008. doi:10.1583/07-2255.1.

22. **Drake JC, Wilson RJ, Yan Z.** Molecular mechanisms for mitochondrial adaptation to exercise training in skeletal muscle. *FASEB J* 30: 13–22, 2016. doi:10.1096/fj.15-276337.

23. **Eastlack RK, Groppo ER, Hargens AR, Pedowitz RA.** Ischemic-preconditioning does not prevent neuromuscular dysfunction after ischemia-reperfusion injury. *J Orthop Res* 22: 918–923, 2004. doi:10.1016/j.orthres.2003.10.015.

24. **Egan B, Zierath JR.** Exercise metabolism and the molecular regulation of skeletal muscle adaptation. *Cell Metab* 17: 162–184, 2013. doi:10.1016/j.cmet.2012.12.012.

25. **Ferraro E, Giammarioli AM, Chiandotto S, Spoletini I, Rosano G.** Exercise-induced skeletal muscle remodeling and metabolic adaptation: redox signaling and role of autophagy. *Antioxid Redox Signal* 21: 154–176, 2014. doi:10.1089/ars.2013.5773.

26. **French JP, Hamilton KL, Quindry JC, Lee Y, Upchurch PA, Powers SK.** Exercise-induced protection against myocardial apoptosis and necro-

sis: MnSOD, calcium-handling proteins, and calpain. *FASEB J* 22: 2862–2871, 2008. doi:10.1096/fj.07-102541.

27. **Fuller W, Parmar V, Eaton P, Bell JR, Shattock MJ.** Cardiac ischemia causes inhibition of the Na/K ATPase by a labile cytosolic compound whose production is linked to oxidant stress. *Cardiovasc Res* 57: 1044–1051, 2003. doi:10.1016/S0008-6363(02)00810-6.

28. **Gillon A, Sheard P.** Elderly mouse skeletal muscle fibres have a diminished capacity to upregulate NCAM production in response to denervation. *Biogerontology* 16: 811–823, 2015. doi:10.1007/s10522-015-9608-6.

29. **Gomez-Cabrera MC, Domenech E, Viña J.** Moderate exercise is an antioxidant: upregulation of antioxidant genes by training. *Free Radic Biol Med* 44: 126–131, 2008. doi:10.1016/j.freeradbiomed.2007.02.001.

30. **Gores GJ, Herman B, Lemasters JJ.** Plasma membrane bleb formation and rupture: a common feature of hepatocellular injury. *Hepatology* 11: 690–698, 1990. doi:10.1002/hep.1840110425.

31. **Handschin C, Kobayashi YM, Chin S, Seale P, Campbell KP, Spiegelman BM.** PGC-1α regulates the neuromuscular junction program and ameliorates Duchenne muscular dystrophy. *Genes Dev* 21: 770–783, 2007. doi:10.1101/gad.1525107.

32. **Huerta-Alardín AL, Varon J, Marik PE.** Bench-to-bedside review: Rhabdomyolysis—an overview for clinicians. *Crit Care* 9: 158–169, 2004. doi:10.1186/cc2978.

33. **Itoh M, Shimokawa N, Tajika Y, Murakami T, Aotsuka N, Lesmana R, Farenia R, Iwasaki T, Okda J, Yorifuji H, Koibuchi N.** Alterations of biochemical marker levels and myonuclear numbers in rat skeletal muscle after ischemia-reperfusion. *Mol Cell Biochem* 373: 11–18, 2013. doi:10.1007/s11010-012-1470-0.

34. **Ji LL.** Exercise-induced modulation of antioxidant defense. *Ann N Y Acad Sci* 959: 82–92, 2002. doi:10.1111/j.1749-6632.2002.tb02085.x.

35. **Ji LL, Kang C, Zhang Y.** Exercise-induced hormesis and skeletal muscle health. *Free Radic Biol Med* 98: 113–122, 2016. doi:10.1016/j.freeradbiomed.2016.02.025.

36. **Kalliainen LK, Jejurikar SS, Liang LW, Urbanchek MG, Kuzon WM Jr.** A specific force deficit exists in skeletal muscle after partial denervation. *Muscle Nerve* 25: 31–38, 2002. doi:10.1002/mus.1216.

37. **Kam PC, Kavanaugh R, Yoong FF.** The arterial tourniquet: pathophysiological consequences and anaesthetic implications. *Anaesthesia* 56: 534–545, 2001. [Erratum in: *Anaesthesia* 56: 821, 2001.] 10.1046/j.1365-2044.2001.01982.x.

38. **Khalil AA, Aziz FA, Hall JC.** Reperfusion injury. *Plast Reconstr Surg* 117: 1024–1033, 2006. doi:10.1097/01.prs.0000204766.17127.54.

39. **Lai L-N, Zhang X-J, Zhang X-Y, Song L-H, Guo C-H, Lei J-W, Song X-L.** Lazaroid U83836E protects the heart against ischemia reperfusion injury via inhibition of oxidative stress and activation of PKC. *Mol Med Rep* 13: 3993–4000, 2016. doi:10.3892/mmr.2016.5030.

40. **Laker RC, Drake JC, Wilson RJ, Lira VA, Lewellen BM, Ryall KA, Fisher CC, Zhang M, Saucerman JJ, Goodyear LJ, Kundu M, Yan Z.** Ampk phosphorylation of Ulk1 is required for targeting of mitochondria to lysosomes in exercise-induced mitophagy. *Nat Commun* 8: 548, 2017. doi:10.1038/s41467-017-00520-9.

42. **Laker RC, Xu P, Ryall KA, Sujkowski A, Kenwood BM, Chain KH, Zhang M, Royal MA, Hoehn KL, Driscoll M, Adler PN, Wessells RJ, Saucerman JJ, Yan Z.** A novel MitoTimer reporter gene for mitochondrial content, structure, stress, and damage in vivo. *J Biol Chem* 289: 12005–12015, 2014. doi:10.1074/jbc.M113.530527.

43. **Lawler JM, Rodriguez DA, Hord JM.** Mitochondria in the middle: exercise preconditioning protection of striated muscle. *J Physiol* 594: 5161–5183, 2016. doi:10.1113/JP270656.

44. **Ledin H, Aspenberg P, Good L.** Tourniquet use in total knee replacement does not improve fixation, but appears to reduce final range of motion. *Acta Orthop* 83: 499–503, 2012. doi:10.3109/17453674.2012.727078.

45. **Lira VA, Okutsu M, Zhang M, Greene NP, Laker RC, Breen DS, Hoehn KL, Yan Z.** Autophagy is required for exercise training-induced skeletal muscle adaptation and improvement of physical performance. *FASEB J* 27: 4184–4193, 2013. doi:10.1096/fj.13-228486.

46. **Lu W, Sun J, Yoon JS, Zhang Y, Zheng L, Murphy E, Mattson MP, Lenardo MJ.** Mitochondrial protein PGAM5 regulates mitophagic protection against cell necroptosis. *PLoS One* 11: e0147792, 2016. doi:10.1371/journal.pone.0147792.

47. **Mattson MP.** Hormesis defined. *Ageing Res Rev* 7: 1–7, 2008. doi:10.1016/j.arr.2007.08.007.

48. **Mohler LR, Pedowitz RA, Ohara WM, Oyama BK, Lopez MA, Gershuni DH.** Effects of an antioxidant in a rabbit model of tourniquet-

induced skeletal muscle ischemia-reperfusion injury. *J Surg Res* 60: 23–28, 1996. doi:10.1006/jsre.1996.0005.

49. **Mufti SA, Carlson BM, Maxwell LC, Faulkner JA.** The free autografting of entire limb muscles in the cat: morphology. *Anat Rec* 188: 417–429, 1977. doi:10.1002/ar.1091880403.

50. **Niess AM, Simon P.** Response and adaptation of skeletal muscle to exercise–the role of reactive oxygen species. *Front Biosci* 12: 4826–4838, 2007. doi:10.2741/2431.

51. **Nikolaou VS, Chytas D, Babis GC.** Common controversies in total knee replacement surgery: Current evidence. *World J Orthop* 5: 460–468, 2014. doi:10.5312/wjo.v5.i4.460.

52. **Olivecrona C, Blomfeldt R, Ponzer S, Stanford BR, Nilsson BY.** Tourniquet cuff pressure and nerve injury in knee arthroplasty in a bloodless–field: a neurophysiological study. *Acta Orthop* 84: 159–164, 2013. doi:10.3109/17453674.2013.782525.

53. **Olivecrona C, Lapidus LJ, Benson L, Blomfeldt R.** Tourniquet time affects postoperative complications after knee arthroplasty. *Int Orthop* 37: 827–832, 2013. doi:10.1007/s00264-013-1826-4.

54. **Pedowitz RA.** Tourniquet-induced neuromuscular injury. A recent review of rabbit and clinical experiments. *Acta Orthop Scand Suppl* 62: 1–33, 1991. doi:10.3109/17453679109153924.

55. **Perry HM, Huang L, Wilson RJ, Bajwa A, Sesaki H, Yan Z, Rosin DL, Kashatus DF, Okusa MD.** Dynamin-related protein 1 deficiency promotes recovery from AKI. *J Am Soc Nephrol* 29: 194–206, 2018. doi:10.1681/ASN.2017060659.

56. **Pizzimenti S, Toaldo C, Pettazzoni P, Dianzani MU, Barrera G.** The "two-faced" effects of reactive oxygen species and the lipid peroxidation product 4-hydroxynonenal in the hallmarks of cancer. *Cancers (Basel)* 2: 338–363, 2010. doi:10.3390/cancers2020338.

57. **Prasannarong M, Santos FR, Hooshmand P, Hooshmand P, Giovannini FJ, Henriksen EJ.** The lipid peroxidation end-product and oxidant 4-hydroxynonenal induces insulin resistance in rat slow-twitch skeletal muscle. *Arch Physiol Biochem* 120: 22–28, 2014. doi:10.3109/13813455.2013.834937.

58. **Pratt SJP, Shah SB, Ward CW, Kerr JP, Stains JP, Lovering RM.** Recovery of altered neuromuscular junction morphology and muscle function in mdx mice after injury. *Cell Mol Life Sci* 72: 153–164, 2015. doi:10.1007/s00018-014-1663-7.

59. **Pratt SJP, Valencia AP, Le GK, Shah SB, Lovering RM.** Pre- and postsynaptic changes in the neuromuscular junction in dystrophic mice. *Front Physiol* 6: 252, 2015. doi:10.3389/fphys.2015.00252.

60. **Quindry J, French J, Hamilton K, Lee Y, Mehta JL, Powers S.** Exercise training provides cardioprotection against ischemia-reperfusion induced apoptosis in young and old animals. *Exp Gerontol* 40: 416–425, 2005. doi:10.1016/j.exger.2005.03.010.

61. **Quindry JC, Hamilton KL.** Exercise and cardiac preconditioning against ischemia reperfusion injury. *Curr Cardiol Rev* 9: 220–229, 2013. doi:10.2174/1573403X113099990033.

62. **Radak Z, Chung HY, Goto S.** Systemic adaptation to oxidative challenge induced by regular exercise. *Free Radic Biol Med* 44: 153–159, 2008. doi:10.1016/j.freeradbiomed.2007.01.029.

63. **Rattray B, Thompson M, Ruell P, Caillaud C.** Specific training improves skeletal muscle mitochondrial calcium homeostasis after eccentric exercise. *Eur J Appl Physiol* 113: 427–436, 2013. doi:10.1007/s00421-012-2446-1.

64. **Sharma JP, Salhotra R.** Tourniquets in orthopedic surgery. *Indian J Orthop* 46: 377–383, 2012. doi:10.4103/0019-5413.98824.

65. **Sanchez AM, Bernardi H, Py G, Candau RB.** Autophagy is essential to support skeletal muscle plasticity in response to endurance exercise. *Am J Physiol Regul Integr Comp Physiol* 307: R956–R969, 2014. doi:10.1152/ajpregu.00187.2014.

66. **Sanchez AM, Candau R, Raibon A, Bernardi H.** Autophagy, a highly regulated intracellular system essential to skeletal muscle homeostasis—role in disease, exercise and altitude exposure. In: *Muscle Cell and Tissue*, edited by Sakuma K. London: IntechOpen, 2015, chapt. 7, p. 171–199. doi:10.5772/60698.

67. **Sapega AA, Heppenstall RB, Chance B, Park YS, Sokolow D.** Optimizing tourniquet application and release times in extremity surgery. A biochemical and ultrastructural study. *J Bone Joint Surg Am* 67: 303–314, 1985. doi:10.2106/00004623-198567020-00018.

68. **Schoen M, Rotter R, Gierer P, Gradl G, Strauss U, Jonas L, Mittlmeier T, Vollmar B.** Ischemic preconditioning prevents skeletal muscle tissue injury, but not nerve lesion upon tourniquet-induced ischemia. *J Trauma* 63: 788–797, 2007. doi:10.1097/01.ta.0000240440.85673.fc.

69. **Symbiosis SG, Shibamoto T, Kuda Y, Tanida M, Wang M, Kurata Y.** *Exercise Attenuates Ischemia-reperfusion Injury of Nonalcoholic Fatty Liver in OLETF Rat* (Online). www.symbiosisonlinepublishing.com [22 August 2015].

70. **Tai TW, Lin CJ, Jou IM, Chang CW, Lai KA, Yang CY.** Tourniquet use in total knee arthroplasty: a meta-analysis. *Knee Surg Sports Traumatol Arthrosc* 19: 1121–1130, 2011. doi:10.1007/s00167-010-1342-7.

71. **Talbert EE, Smuder AJ, Min K, Kwon OS, Szeto HH, Powers SK.** Immobilization-induced activation of key proteolytic systems in skeletal muscles is prevented by a mitochondria-targeted antioxidant. *J Appl Physiol (1985)* 115: 529–538, 2013. doi:10.1152/japplphysiol.00471.2013.

72. **Tam BT, Siu PM.** Autophagic cellular responses to physical exercise in skeletal muscle. *Sports Med* 44: 625–640, 2014. doi:10.1007/s40279-013-0140-z.

73. **Terskikh A, Fradkov A, Ermakova G, Zaraisky A, Tan P, Kajava AV, Zhao X, Lukyanov S, Matz M, Kim S, Weissman I, Siebert P.** "Fluorescent timer": protein that changes color with time. *Science* 290: 1585–1588, 2000. doi:10.1126/science.290.5496.1585.

74. **Tu H, Zhang D, Corrick RM, Muelleman RL, Wadman MC, Li Y-L.** Morphological Regeneration and functional recovery of neuromuscular junctions after tourniquet-induced injuries in mouse hindlimb. *Front Physiol* 8: 207, 2017. doi:10.3389/fphys.2017.00207.

75. **Vaughan A, Hardwick T, Gaskin J, Bendall S.** Tourniquet use in orthopaedic surgery. *Orthop Trauma* 31: 312–315, 2017. doi:10.1016/j.mporth.2017.07.010.

76. **Webb R, Hughes MG, Thomas AW, Morris K.** The ability of exercise-associated oxidative stress to trigger redox-sensitive signalling responses. *Antioxidants* 6: 63, 2017. doi:10.3390/antiox6030063.

77. **Wilson RJ, Drake JC, Cui D, Lewellen BM, Fisher CC, Zhang M, Kashatus DF, Palmer LA, Murphy MP, Yan Z.** Mitochondrial protein S-nitrosation protects against ischemia reperfusion-induced denervation at neuromuscular junction in skeletal muscle. *Free Radic Biol Med* 117: 180–190, 2018. doi:10.1016/j.freeradbiomed.2018.02.006.

78. **Wilson RJ, Drake JC, Cui D, Zhang M, Perry HM, Kashatus JA, Kusminski CM, Scherer PE, Kashatus DF, Okusa MD, Yan Z.** Conditional MitoTimer reporter mice for assessment of mitochondrial structure, oxidative stress, and mitophagy. *Mitochondrion* S1567-7249(17)30196-4, 2017. doi:10.1016/j.mito.2017.12.008.

79. **Wong MS, Lara TM, Kobzik L, Rounds JD, Robinson MK, Jacobs DO.** Hindlimb ischemia-reperfusion increases complement deposition and glycolysis. *J Surg Res* 85: 130–135, 1999. doi:10.1006/jsre.1999.5657.

80. **Yan Z, Okutsu M, Akhtar YN, Lira VA.** Regulation of exercise-induced fiber type transformation, mitochondrial biogenesis, and angiogenesis in skeletal muscle. *J Appl Physiol (1985)* 110: 264–274, 2011. doi:10.1152/japplphysiol.00993.2010.

81. **Zager RA, Sacks BM, Burkhart KM, Williams AC.** Plasma membrane phospholipid integrity and orientation during hypoxic and toxic proximal tubular attack. *Kidney Int* 56: 104–117, 1999. doi:10.1046/j.1523-1755.1999.00533.x.

82. **Zampieri S, Mammucari C, Romanello V, Barberi L, Pietrangelo L, Fusella A, Mosole S, Gherardi G, Höfer C, Löfler S, Sarabon N, Cvecka J, Krenn M, Carraro U, Kern H, Protasi F, Musarò A, Sandri M, Rizzuto R.** Physical exercise in aging human skeletal muscle increases mitochondrial calcium uniporter expression levels and affects mitochondria dynamics. *Physiol Rep* 4: e13005, 2016. doi:10.14814/phy2.13005.