

Muscle inflammatory cells after passive stretches, isometric contractions, and lengthening contractions

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Pizza, Francis X., Timothy J. Koh, Stephen J. McGregor, and Susan V. Brooks. Muscle inflammatory cells after passive stretches, isometric contractions, and lengthening contractions. *J Appl Physiol* 92: 1873–1878, 2002. First published December 21, 2001; 10.1152/jappphysiol.01055.2001.—We tested the hypotheses that lengthening contractions, isometric contractions, and passive stretches increase muscle inflammatory cells (neutrophils and macrophages) and that prior conditioning with lengthening contractions, isometric contractions, or passive stretches reduces neutrophils and macrophages after subsequent lengthening contractions. Extensor digitorum longus muscles in anesthetized mice were subjected in situ to lengthening contractions, isometric contractions, or passive stretches. Six hours or 3 days after a protocol of contractions or passive stretches, neutrophils and macrophages were quantified in muscle cross sections. Three days after isometric contractions or passive stretches, neutrophils were elevated ($P < 0.05$) 3.7- and 5.5-fold, respectively, relative to controls. Both macrophages and neutrophils were increased 51.2- and 7.9-fold, respectively, after lengthening contractions. Prior lengthening contractions, isometric contractions, or passive stretches reduced inflammatory cells after lengthening contractions performed 2 wk later. The major finding of this study was that passive stretches and isometric contractions elevated neutrophils without causing overt signs of injury. Because both passive stretches and isometric contractions elevated neutrophils and afforded some protection from contraction-induced muscle injury, neutrophils and/or the related inflammatory events may contribute to the induction of a protective mechanism.

neutrophils; macrophages; muscle injury; muscle degeneration

LENGTHENING CONTRACTIONS CAUSE focal sarcomeric disruptions, altered sarcolemma permeability, muscle soreness, and a loss in joint range of motion and muscular strength (reviewed in Refs. 3, 5). Changes in these markers of muscle injury are reduced after a second bout of lengthening contractions, indicating an adaptation that protects skeletal muscle from subsequent injury (3, 10, 21, 25, 26, 32). Although several mechanisms have been proposed, including increased sarcomere number (13) and a greater homogeneity in

sarcomere strength during contraction (4), the adaptations responsible for protection from muscle injury are not well understood (18).

One aspect of the adaptation to muscle injury that has yet to be fully characterized is the response of inflammatory cell populations (25, 26). Specifically, muscle neutrophil and macrophage concentrations have not been quantified after repeated bouts of lengthening contractions. Neutrophils, the first inflammatory cell type to appear in injured muscle (6, 22, 23, 37), have been suggested to both impair and aid events associated with muscle regeneration. Although no direct in vivo evidence exists, neutrophils have been hypothesized to delay muscle regeneration by exacerbating the initial injury and/or by injuring myotubes through the release of free radicals and proteases (27). Alternatively, neutrophils could facilitate muscle regeneration by removing tissue debris from the injured area via phagocytosis (23) and by activating satellite cells (29, 34). Macrophages, which increase in concentration 1–3 days after injury (6, 22, 35, 37), are thought to contribute to muscle regeneration (reviewed in Refs. 7, 36). The beneficial contribution of macrophages to the events associated with muscle regeneration has been ascribed to their ability to phagocytose tissue debris (20, 23) and to their capacity to cause myoblast proliferation in vitro (1, 14, 19, 28).

Recently, Koh and Brooks (10) reported that, in addition to lengthening contractions, prior passive stretches or isometric contractions afforded some protection against lengthening contraction-induced muscle injury. Because neither passive stretches nor isometric contractions caused overt morphological damage or an isometric force deficit, the conclusion was that muscle degeneration was not necessary to induce the protective adaptation (10). If passive stretches and isometric contractions increase muscle inflammatory cells in the absence of overt injury, this could provide a potential mechanism for inducing protection. Therefore, we tested the hypotheses that lengthening contractions, passive stretches, and isometric contractions increase muscle inflammatory cell concentrations and that prior performance of lengthening contractions, isometric

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contractions, or passive stretches reduces muscle inflammatory cells after subsequent lengthening contractions.

METHODS

Animals. Three- to four-month-old adult male C57BL/6 mice ($n = 71$, 27.0 ± 0.4 g; Harlan Sprague Dawley, Indianapolis, IN) were housed in a specific pathogen-free environment. Experimental procedures were approved by the University Committee for the Use and Care of Animals at the University of Michigan. Most of the muscle sections used in the present study were taken from mice in a previous study (10).

In situ muscle preparation. The muscle preparation procedures were described by Koh and Brooks (10). Briefly, the mice were anesthetized with an intraperitoneal injection of 2% avertin (0.015 ml/g body mass) with supplemental doses (0.1 ml) being administered if the mouse responded to a toe pinch. While the animal was under anesthesia, the distal tendon of the extensor digitorum longus (EDL) was exposed and tied to the lever arm of a servomotor (Aurora Scientific, Richmond Hill, Ontario), which controlled the length of the muscle and measured the force generated by the muscle. Activation of the EDL muscles was accomplished by stimulating the peroneal nerve with needle electrodes (model S88, Grass Instruments, West Warwick, RI). Pulse duration was kept constant (0.2 ms), whereas pulse intensity, frequency, and optimal muscle length (L_o) for isometric force development were determined separately for each animal, as previously described by Koh and Brooks (10). Optimal fiber length (L_f) was determined by multiplying L_o by the L_f -to- L_o ratio of 0.44 (17).

Experimental treatments. Mice were divided into groups that were administered a single bout of lengthening contractions, passive stretches, or isometric contractions or to groups that performed a bout of lengthening contractions, passive stretches, or isometric contractions followed by a bout of lengthening contractions 2 wk later. The lengthening contraction protocols consisted of lengthening through 20% strain relative to L_f with the muscles stimulated at 150 Hz. The protocol of passive stretches was identical to the protocol of lengthening contractions, except muscles were not stimulated. The protocol of isometric contractions consisted of stimulation at 150 Hz while the EDL muscles were held at L_o . Each protocol involved 75 repetitions performed at 0.25 Hz for a total exercise duration of 5 min. Mice that had normal cage activity and mice that underwent identical surgical procedures without exercise served as controls. Controls for the second bout of lengthening contractions performed a single bout of lengthening contractions and were killed 2 wk later. Other mice that received sham surgical treatment were subjected to lengthening contractions 2 wk later to determine whether surgical treatment alone provided protection from injury. Mice were killed via cervical dislocation under anesthesia at either 6 h or 3 days after the final in situ procedure.

Immunohistochemistry of inflammatory cells. The EDL muscles were excised with tendons intact, coated with optimum cutting temperature compound, frozen in melting isopentane cooled on dry ice, and stored at -70°C . Cross sections (10 μm) were cut from the midbelly of the muscles and were prepared for immunohistochemistry by being fixed in cold acetone and quenched with hydrogen peroxide (6). Neutrophils were identified with an anti-mouse Ly6G antibody (1:100 in PBS; PharMingen, Franklin Lake, NJ) (8, 12), whereas macrophages were recognized by using an anti-F4/80 antibody (1:100 in PBS; Serotec, Raleigh, NC) (9).

Slides serving as negative controls received PBS instead of primary antibody. After 3-h incubation at room temperature with the primary antibody, sections were washed in PBS, incubated with biotinylated mouse absorbed anti-rat IgG (1:200 in PBS; Vector Laboratories, Burlingame, CA), followed by avidin D horseradish peroxidase (1:1,000 in PBS). Sections were then developed with 3-amino-9-ethylcarbazole (Vector Laboratories).

Sections were viewed with a light microscope (Olympus IX-70; Olympus, Melville, NY) with Nomarski optics. The number of inflammatory cells in two entire sections for each muscle was manually counted, and the total area of the section was measured with a calibrated square grid. The volume of muscle sampled was calculated as the product of the cross-sectional area of the section and the section thickness (10 μm). Inflammatory cells were expressed as number per cubic millimeter (mm^3). The number of fibers invaded by neutrophils or macrophages was also counted and expressed as a percentage of the total number of fibers within the section.

Statistical analyses. The effect of passive stretches and isometric contractions and the influence of their prior performance on inflammatory cells after lengthening contractions were determined with separate one-way ANOVA tests (SigmaStat; Sigma Chemical, St. Louis, MO). Parametric statistics were performed on these analyses because inflammatory cell data passed tests of normality and equal variance (SigmaStat). A two-way ANOVA was used to determine the effect of repeated bouts of lengthening contractions. The Newman-Keuls post hoc test was used to locate the differences between means when the observed F ratio was statistically significant ($P < 0.05$). Data are reported as means \pm SE.

RESULTS

Both passive stretches and isometric contractions increased neutrophils at 3 days by 5.5- ($P = 0.001$) and 3.7-fold ($P = 0.019$), respectively, relative to controls (Fig. 1A). The increase in neutrophils after either passive stretches or isometric contractions was only about one-half as large as the 7.9-fold increase observed 3 days after lengthening contractions ($P < 0.001$). Interestingly, macrophage concentrations were elevated 51.2-fold at 3 days after lengthening contractions ($P = 0.002$) but were not significantly elevated after passive stretches or isometric contractions (Fig. 1B). The observed changes were not attributable to surgical procedures because inflammatory cells were not elevated in sham surgical controls (data not reported).

Neutrophils were elevated relative to controls at 6 h and 3 days for each of the two bouts of lengthening contractions separated by 2 wk and were higher at 3 days relative to 6 h (time effect; $P < 0.001$; Fig. 2A). Although both bouts of lengthening contractions increased neutrophils, the concentrations observed after the second bout were $\sim 40\%$ lower than the levels observed after the first bout (bout effect; $P = 0.02$; Fig. 2A). The percentage of fibers invaded by neutrophils at 3 days was elevated for both bouts relative to controls (time effect; $P < 0.001$) but was 45% lower for the second bout of lengthening contractions relative to the first bout (interaction; $P = 0.03$; Fig. 2B). Although the effect of repeated bouts of lengthening contractions on macrophages was similar to that observed for neutro-

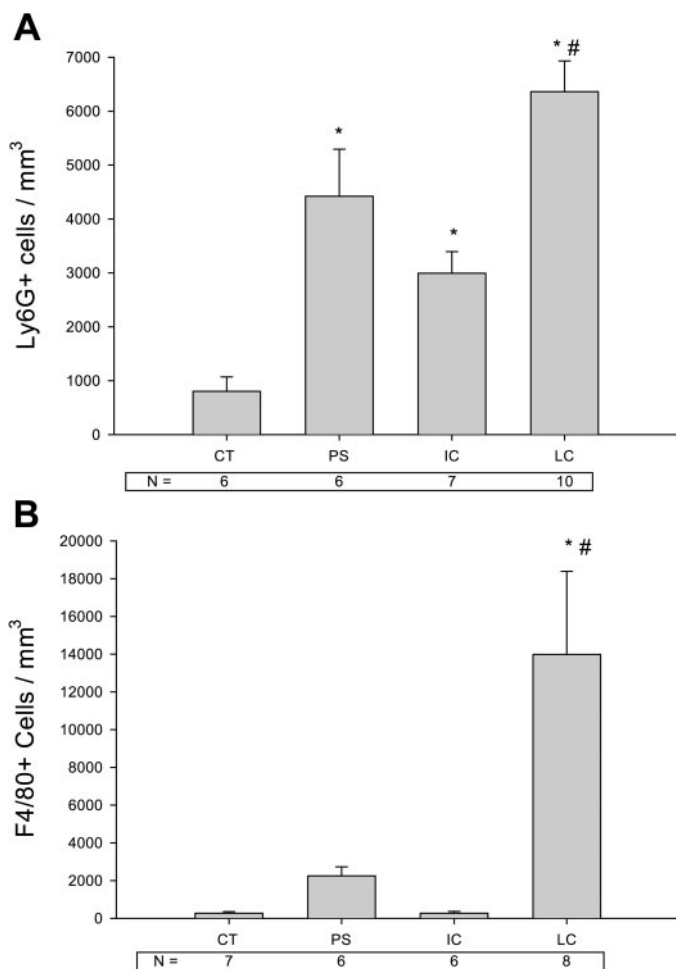


Fig. 1. Neutrophil (Ly6G+ cells) concentrations (A) and macrophage (F4/80+ cells) concentrations (B) in the extensor digitorum longus (EDL) muscles 3 days after a single bout of exercise. CT, controls; PS, passive stretches; IC, isometric contractions; LC, lengthening contractions. Values are means \pm SE; N, no. of mice. *Significantly different relative to CT; # significantly different from PS and IC: $P < 0.05$.

phils, the reduction in macrophages after the second bout was more dramatic than the decrease observed for neutrophils. The concentration of macrophages and the percentage of fibers invaded by macrophages were 78% ($P = 0.027$) and 94% ($P = 0.003$) lower, respectively, at 3 days after the second bout of lengthening contractions relative to the first bout (interaction). Furthermore, despite increases in the concentration of macrophages (Fig. 3A) and the percentage of fibers invaded by macrophages (Fig. 3B) at 3 days after the first bout of lengthening contractions, neither was significantly elevated after the second bout (Fig. 3). The resolution of muscle inflammation after the first bout of lengthening contractions was complete by 2 wk, as indicated by similarities in inflammatory cell concentrations between controls for the first and second bout of lengthening contractions (Figs. 2A and 3A). Prior conditioning with either passive stretches or isometric contractions was as effective as a bout of lengthening contractions at reducing neutrophils (Fig. 4A) and macrophages (Fig.

4B) after lengthening contractions administered 2 wk later. The significant reductions in both neutrophils and macrophages for the second bout of lengthening contractions were not attributable to the surgical procedures because sham surgery performed 2 wk before lengthening contractions did not reduce inflammatory cells (data not reported).

DISCUSSION

The major novel observation of the present study was the elevation in neutrophils resulting from exposure to a protocol of either passive stretches or isometric contractions, protocols that did not result in overt histological or functional signs of injury (10). Because both passive stretches and isometric contractions elevated neutrophils and afforded some protection against lengthening contraction-induced muscle injury

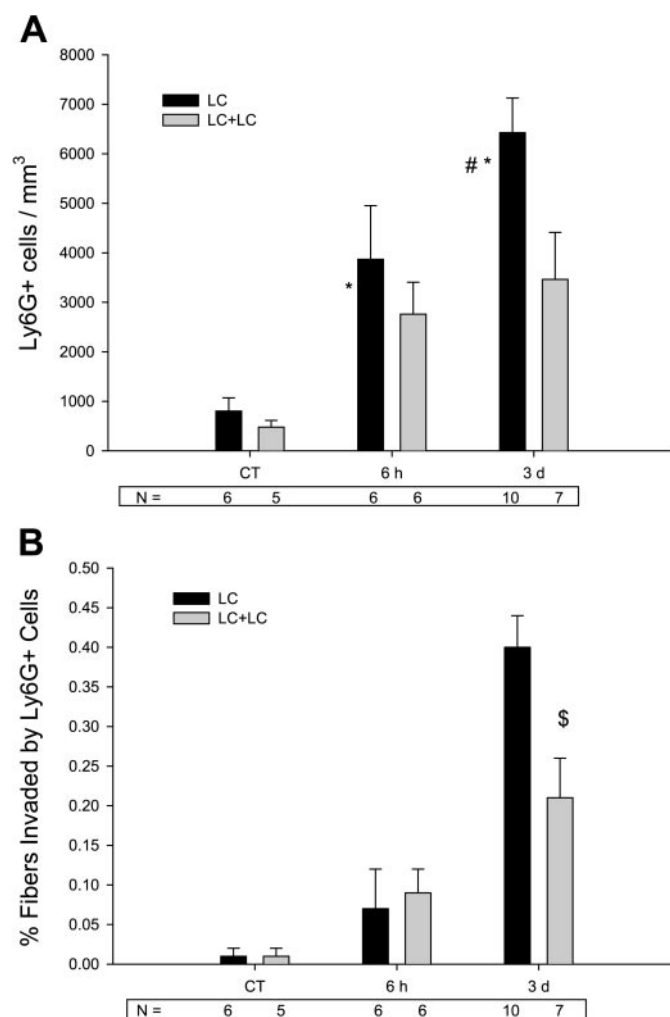


Fig. 2. Neutrophil (Ly6G+ cells) concentrations (A) and percentage of fibers invaded by neutrophils (B) in the EDL muscles after bouts of lengthening contractions. Values are means \pm SE; N, no. of mice. #Significantly higher for LC relative to the same contractions performed 2 wk later (LC+LC; bout effect); *significant time effect for both LC and LC+LC relative to CT; \$significantly elevated for both LC and LC+LC relative to controls and significantly lower for LC+LC relative to LC (interaction): $P < 0.05$.

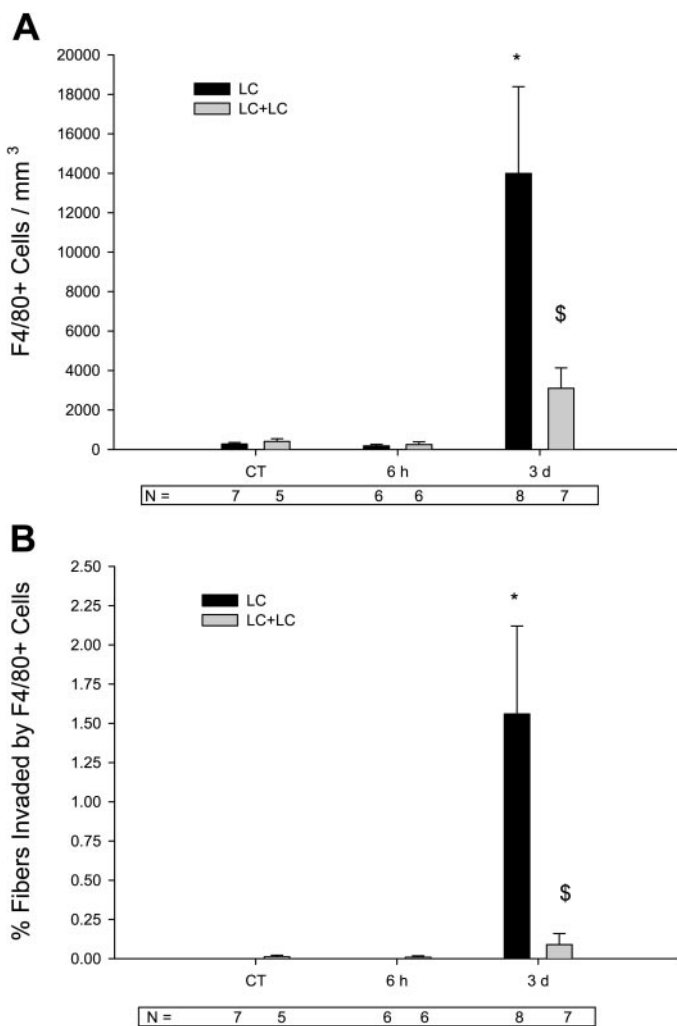


Fig. 3. Macrophage (F4/80+ cells) concentrations (A) and percentage of fibers invaded by macrophages (B) in the EDL muscles after bouts of lengthening contractions. Values are means ± SE; N, no. of mice. *Significant time effect for LC relative to controls; §significant difference between bouts at 3 days (interaction); *P* < 0.05.

(10), neutrophils and/or related inflammatory events may contribute to the mechanism for protection from injury.

The majority of studies that have quantified skeletal muscle inflammatory cells have used hindlimb suspension reloading (6, 37) and traumatic injury models (22, 23), whereas St. Pierre Schneider et al. (35) quantified muscle macrophages after lengthening contractions. The significant increase in macrophages after a single bout of lengthening contractions in the present study, which occurred at a time when the muscles exhibited a 55% isometric force deficit and 18% of the fibers showed overt evidence of damage (10), is consistent with responses observed after other models of overt muscle injury (6, 22, 35, 37). Surprisingly, macrophages were not elevated 3 days after a second bout of lengthening contractions, despite a 20% isometric force deficit and 8% of the fiber showing evidence of overt injury (10). These data may indicate that macrophages

increase in muscle only when substantial signs of overt injury are apparent.

Neutrophils, although lower after the second bout of lengthening contractions relative to the first bout, were elevated after both bouts of lengthening contractions. In addition, neutrophils were increased by passive stretches and isometric contractions. The elevation in neutrophils after passive stretches and isometric contractions occurred when the muscles showed no overt histological or functional signs of injury (10). The elevated neutrophils, in contrast to macrophages, may indicate that muscle neutrophils are increased by non-injurious, as well as injurious, muscle activity. One caveat to this interpretation is that some minor injury (i.e., injury that does not result in a functional impairment or gross histological disruptions) may have occurred after passive stretches and isometric contractions, and thus one or more chemoattractant(s) for neutrophils may have been produced and/or released. Alternatively, mere activation and/or mechanical loading of skeletal muscle may cause the release of chemoattractants for neutrophils. Because of the novelty

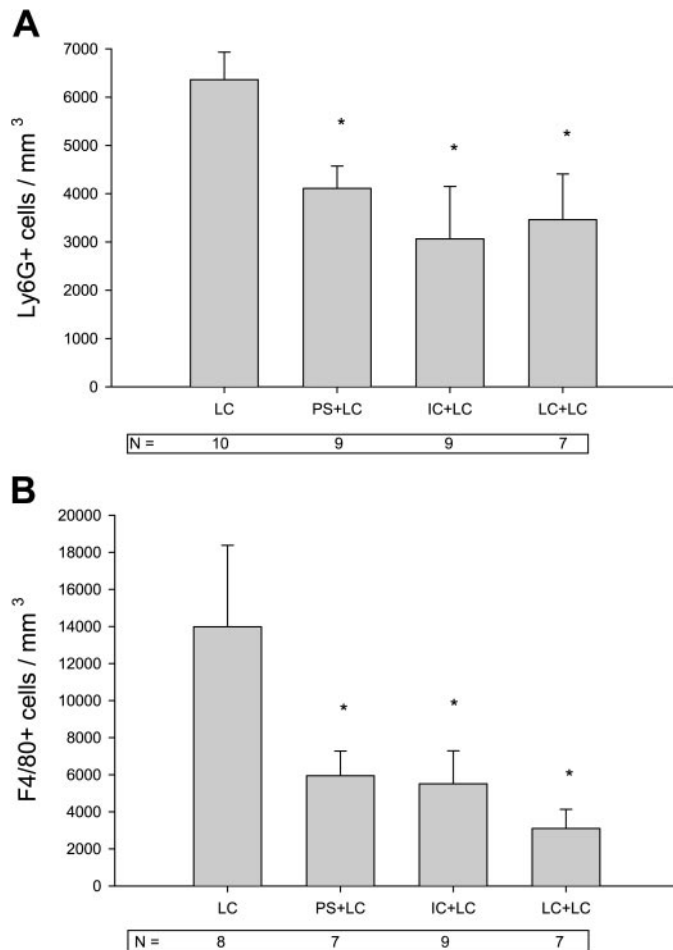


Fig. 4. Neutrophil (Ly6G+ cells) concentrations (A) and macrophage (F4/80+ cells) concentrations (B) in the EDL muscles conditioned by LC, PS, or IC. LC, PS, or IC conditioning was performed 2 wk before LC (LC+LC, PS+LC, IC+LC, respectively). Values are means ± SE; N, no. of mice. *Significantly different relative to LC, *P* < 0.05.

of our observations and the myriad of factors known to cause inflammatory cell chemotaxis (reviewed in Refs. 7, 15), it is difficult to speculate, with confidence, on chemoattractants that may have been produced and/or released after passive stretches or isometric contractions that attracted neutrophils but not macrophages. One possibility for neutrophil chemotaxis, however, involves superoxide anion, a reactive oxygen species. McArdle et al. (16) have recently reported that muscle contractions, which did not cause overt injury, increased muscle-derived superoxide anion production. Superoxide anion and the downstream reaction product hydrogen peroxide have been reported to cause oxidative modification of plasma proteins that cause neutrophil chemotaxis (24) and enhance neutrophil activation (i.e., reactive oxygen species production and degranulation) (11), respectively. Whether superoxide anion causes oxidative modification of plasma proteins and/or skeletal muscle proteins that serve as chemoattractants for neutrophils after increased muscle use is an intriguing possibility that has yet to be investigated.

Inflammatory cells could contribute to the adaptation to muscle injury either by influencing degenerative and/or regenerative events after overt injury or by providing cellular signals for the induction of a protective mechanism in the absence of overt injury. The observed elevation in neutrophils after passive stretches and isometric contractions, which, when performed 2 wk before a bout of lengthening contractions afforded some protection against lengthening contraction-induced muscle injury (10), suggest that neutrophils and/or the related inflammatory events may contribute to the induction of a protective mechanism. Granted, passive stretches and isometric contractions cause numerous metabolic, molecular, and cellular changes that could contribute to the mechanism for the protection, changes that may not be related to neutrophils. However, neutrophil-derived free radicals, proteases, growth factors, cytokines, and chemokines (reviewed in Refs. 2, 30, 33) could function as signals for the induction of a protective mechanism.

Although prolonged (e.g., 1–2 h) ischemia followed by reperfusion causes neutrophils to injure skeletal muscle (reviewed in Ref. 31), the contribution of neutrophils to the degenerative and regenerative events after overt injury induced by lengthening contractions or muscle trauma is poorly understood. Based on their ability to perform phagocytosis (reviewed in Ref. 30) and on limited qualitative observations (23), neutrophils are thought to contribute to the phagocytosis of tissue debris after overt muscle injury. Recent evidence, however, indicates that neutrophils may have additional effects in regenerating muscle by injuring myotubes (27) or by activating satellite cells (34). The novel observation in the present study that neutrophils are elevated after protocols of either passive stretches or isometric contractions may indicate that neutrophils also contribute to events associated with noninjurious muscle activity. These observations warrant further investigation into the function of neutrophils within

skeletal muscle and into factors that attract neutrophils to injured and noninjured skeletal muscle. Because overt injury is not required for inducing protection from lengthening contraction-induced muscle injury (10), our observations also warrant a study that determines the contribution of neutrophils to the mechanism for protection from injury.

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