Acute phase response in exercise
III. Neutrophil and IL-1β accumulation in skeletal muscle

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—Nine untrained men (22-29 yr) performed 45 min of downhill running (16% incline, 70% of maximum heart rate). Needle biopsies of the vastus lateralis were performed before, 45 min after, and 5 days after exercise. Immunohistochemical staining of muscle cross sections revealed a 135% increase in muscle interleukin-1β (IL-1β) immediately after and a 250% increase (P < 0.03) 5 days after exercise. Using a rating scale (0-3) for the presence of neutrophils, light microscopic examination showed a significant accumulation of neutrophils in muscle biopsies taken 45 min after and 5 days after exercise (before: 0.5 ± 0.2, 45 min after: 1.5 ± 0.3 (P < 0.01), and 5 days after: 1.2 ± 0.2 (P < 0.04)). In addition, electron microscopic analysis showed an increase in the percentage of damaged Z-bands relative to total Z-bands (before: 4.8 ± 3.5%, 45 min after: 32.5 ± 8.6% (P < 0.05), and 5 days after: 14.1 ± 3.2%). Neutrophil accumulation was positively correlated to intracellular Z-band damage (r = 0.66, P < 0.001). Immunohistochemical staining for IL-1β was related to neutrophil accumulation in muscle (r = 0.38, P < 0.06) and to plasma creatine kinase levels (r = 0.416, P < 0.04). These data indicate that after eccentric exercise ultrastructural damage to skeletal muscle is associated with neutrophil infiltration and muscle IL-1β accumulation.

interleukin-1β; Z-band damage; neutrophil; microscopy

unaccustomed physical activity is associated with delayed onset muscle soreness (18) and myofibrillar damage (15, 19). Eccentric muscle actions during exercise are thought to be a major contributor to this delayed soreness and muscle injury (15). Eccentric muscle contractions occur when the muscle develops force as it is being lengthened by an external force. Activities known to have a large eccentric component (e.g., downhill running) are associated with alterations in muscle ultrastructure such as Z-band streaming and loss of contractile proteins (15, 23). Other reactions induced by this exercise are similar to the so-called “acute phase response” to infection, including mobilization and redistribution of neutrophils and increased proteolysis. It is believed that this series of coordinated processes is mediated by cytokines such as interleukin-1β (IL-1β; Ref. 9). After a single session of eccentric exercise, plasma levels of myocellular enzymes increase (11), circulating neutrophil numbers increase (6), muscle and plasma levels of IL-1β rise (3), and myofibrillar and whole body proteolysis are elevated for several days (13).

In previous studies of systemic events we have observed a correlation between exercise-induced muscle enzyme leakage and neutrophil mobilization and activation after 45 min of downhill running (6). Neutrophils may be involved in exercise-induced muscle reactions because of their ability to invade host tissues, generate superoxide (1), and release proteases (21). After 45 min of downhill running, a relationship was observed between peripheral blood mononuclear cell production of IL-1β and myofibrillar protein degradation as measured by urinary 3-methylhistidine excretion (5). Several studies have reported that IL-1β and tumor necrosis factor (TNF)-α act synergistically to induce specifically myofibrillar proteolysis (14, 26). To extend the findings of these previous studies, which relied on systemic measures of muscle damage, cytokines, and neutrophils, the purpose of the present study was to examine the relationships between these factors within muscle tissue.

METHODS

Human subjects protocol. Nine healthy sedentary young men (22-29 yr) were recruited for this study. As part of a larger investigation, four of the subjects supplemented their diets with DL-α-tocopherol (vitamin E) taken as capsules (400 IU in soybean oil, twice daily, Hoffman-La Roche, Nutley, NJ) for 48 days before the exercise. The other subjects ingested a soybean oil placebo for the same period of time. The parameters in this report were not significantly affected by vitamin E supplementation. Therefore, data from the vitamin E and placebo groups have been combined. The physiological characteristics of the subjects have been reported in detail previously (6).

Before acceptance into the study, each subject had a complete physical examination and successfully performed a test of his maximum oxygen uptake (V̇O₂max) on a cycle ergometer. The exercise session consisted of three 15-min bouts of downhill treadmill running on a negative 16% incline, each separated by a 5-min rest period. The exercise intensity was set at 75% of each subject’s maximum heart rate determined by the V̇O₂max test performed several months earlier. Percutaneous needle biopsies of the vastus lateralis were performed before, 45 min after, and 5 days after the exercise. Biopsies obtained on the day of exercise were obtained from separate incisions made in the same leg. The biopsy obtained on day 5 was taken from the opposite leg. Blood samples were obtained before, immediately after, 3 and 6 h after, and 1, 2, 5, and 12 days after exercise. The subjects ate a self-selected meat-free diet during the study period and were instructed to refrain from physical exercise, vitamin supplements, and all anti-inflammatory or analgesic drugs throughout the course of the study.

Muscle biopsy procedures. Biopsies were performed under local anesthetic (1% Xylocaine HCl) using a 5-mm Duchenne needle with applied suction (12). The tissue was divided into three pieces. One piece was quick frozen in liquid nitrogen. The second piece was oriented longitudinally, mounted in embed-
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During medium (OCT, Miles Laboratories, Naperville, IL), and frozen in isopentane cooled to the temperature of liquid nitrogen. Within 5 min of obtaining the biopsy material, the third piece was finely minced and fixed in 0.1 M cacodylate-buffered glutaraldehyde for 3 h at 4°C, rinsed in cacodylate buffer, and postfixed in 1% osmium tetroxide.

**Immunohistochemistry.** Transverse sections of the muscle biopsies were stained immunohistochemically with ammonium sulfate-precipitated rabbit antisera raised against human recombinant IL-1β and with non-immune control rabbit serum (3). Serial 8-μm cryostat sections were affixed to gelatinized cover slips and then fixed for 30 min with 4% paraformaldehyde in 0.1 M phosphate-buffered 0.9% saline (PBS), pH 7.4, containing 0.1 M sucrose. Muscle sections were incubated sequentially in the following solutions: PBS containing 3% H₂O₂ and 0.24% Triton X-100 for 30 min at room temperature; PBS containing 5% nonfat dried milk (PBSM) for 60 min at room temperature; primary antisera diluted 1:500 with PBSM overnight at 4°C; goat anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (Atlantic Antibodies, Scarborough, ME) diluted 1:500 in PBSM for 60 min at room temperature; and PBS containing 0.05% 3,3’-diaminobenzidine (Sigma, St. Louis, MO) and 0.01% H₂O₂ for 10 min at room temperature. Between each incubation step, the muscle sections were rinsed five times with PBS. Preparation and characterization details have been reported previously for the polyclonal rabbit antiserum raised against recombinant human IL-1β (3). Previously, we reported that no increased immunoreactivity occurred when sections were stained with either anti-IL-1α or anti-TNF-α. In addition, specimens incubated with non-immune rabbit serum

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**Fig. 1.** Bright-field photomicrograph of muscle biopsy obtained 45 min postexercise (×33, A; ×200, B). This section was ranked as a grade 3. Note the numerous neutrophils accumulated around and within the muscle fibers.
or with anti-IL-1β that had been preabsorbed with 50 μg/ml of recombinant human IL-1β exhibited little staining (3). Muscle extracts that stained positively for IL-1β also augmented T-cell proliferation, a biological activity of IL-1 (3).

Quantitative image analysis of muscle fibers. Transverse sections stained for IL-1β were analyzed using an image analysis system consisting of a Hamamatsu monochrome video camera mounted on a Zeiss light microscope (Carl Zeiss, Thornwood, NY) and coupled to a Macintosh IIfx computer (Apple Computer, Cupertino, CA) through a Data Translation video capture board (Data Translation Systems, Marlboro, MA). In preliminary studies, serial sections stained with non-immune serum or anti-IL-1β serum were compared, and the region of a 256 unit grey scale that corresponded to specific IL-1β staining was determined. Then, by viewing at 100X and analyzing areas of 40,000 pixels (0.084 mm²), the percentage of pixels within this gray scale range was calculated per square millimeter using software developed at the National Institutes of Health by Dr. W. Rasband (Image 1.2). At minimum, six fields per sample with ~40 muscle fibers per field were analyzed.

Light and electron microscopy. After rinsing, ethyl alcohol was used for dehydration and propylene oxide for infiltration. Embedding was carried out in 1X-112. Between 6 and 12 samples per subject were orientated and flat embedded. Semithin sections (1 μm) were stained with methylene blue, basic fuchsin, and azure blue. Approximately 75 fibers from each biopsy were examined with a light microscope. The same blocks that were examined with a light microscope were thin sectioned for electron microscopic examination. The sections were stained with uranyl acetate and lead citrate and examined with a Zeiss EM-10CA electron microscope (Carl Zeiss).

Quantification of light and electron micrographs. Light microscopic examination of thick sections included criteria for assessing neutrophil aggregation. A rating scale of 0–3 was employed for each section analyzed: 0: no neutrophils; 1: between 1 and 10 neutrophils observed per 20 fibers with neutrophils observed scattered around fibers and not in dense populations; 2: neutrophils observed surrounding at least 25% of the fiber population with evidence of some neutrophils within interstitial spaces; 3: neutrophils observed within interstitial space and within at least one fiber). A blinded evaluation of each section was performed by two separate investigators. Results from both examiners were comparable so an average score was determined for each section. A minimum of 6 sections per time point were rated and the average score obtained. An average of 99 cross-sectional, longitudinal, and oblique fibers were studied per subject.

An average of eight (range 5–12) longitudinally oriented fibers per time point were analyzed from electron micrographs printed at a final magnification of ×12,000. Grid coordinates of individual fibers were recorded before taking pictures to assure that no fiber was analyzed more than once. Stereological measurements of volume densities of skeletal muscle fibers, Z-bands, damaged Z-bands, and focal damage were made with a 100-point isotropic semicircular test system. The test grid points approximated those used by other investigators for estimation of volume densities in skeletal muscle (10, 16). Intersection counts were obtained with the test lines oriented at an angle of 19⁰ to the direction of the longitudinal axis of the muscle fiber. The ratio of damaged to total Z-bands was computed for each time point. In addition, stereological analysis of focal damage was assessed with the same grid system. Focal damage was defined as an area falling on a test grid point showing absent or disorganized myofilaments not associated with Z-band damage.

**Plasma creatine kinase and urinary creatinine excretion.**

These analyses were carried out using a Cobas Fara II automated centrifugal analyzer and reagent kits (Roche Diagnostic systems, Nutley, NJ). Creatine kinase (EC 2.7.3.2) activity was measured as the catalytic transfer of a phosphate to ADP. The rate of ATP formation was detected through coupled reactions resulting in the reduction of NADP to NADPH, which was measured spectrophotometrically at 340 nm. Urinary creatinine was measured reacted with picric acid, and the rate of red complex formation was measured at 520 nm.

**Statistical analysis.** Data were analyzed using the Statview statistical package (Abacus concepts). All values are reported as means ± SE. Significant differences between time points were determined by one-way analysis of variance followed by Fisher’s least significant difference test. Associations between variables were investigated using the Spearman rank correlation coefficient (r). Significance was set at P < 0.05.

**RESULTS**

**Microscopy.** Increased neutrophil infiltration was observed immediately after and 5 days after exercise. Using the 0–3 scale, neutrophils increased significantly [before: 0.54 ± 0.03; 45 min after: 1.56 ± 0.34 (P < 0.01); 5 days after: 1.26 ± 0.26 (P < 0.05)]. Figure 1, A and B, shows the same light micrograph rated grade 3 from a biopsy obtained 45 min after exercise (A: ×33; B: ×200). Electron microscopic stereological analysis revealed a significant increase in the ratio of damaged to total Z-bands. The ratio increased from 4.8 ± 3.5% of total Z-band volume before exercise to 32.5 ± 8.6% (P < 0.01) immediately after exercise, and it returned to 14.1 ± 3.2% 5 days after exercise (Fig. 2). Figures 3 and 4 show representative electron micrographs from a subject taken 45 min after exercise (with the anisotropic test grid superimposed). Figure 3 shows extensive Z-band damage, and Fig. 4 shows small areas of focal damage with little or no Z-band disruption. A significant correlation was noted between neutrophil infiltration within the muscle samples and the ratio of damaged to total Z-bands (r = 0.687, P < 0.001).

In addition, stereological analysis of focal damage revealed an increase in the volume of focal damage from 0.2 ± 1.3 to 6.2 ± 3.4% (P < 0.05) 45 min after exercise that returned to baseline 5 days after exercise (5.5 ± 1.6%). A positive correlation was obtained between the volume percentage of focal damage and the ratio of damaged to total Z-bands (r = 0.680, P < 0.001).

**IL-1β.** Muscle levels of IL-1β increased after exercise, reaching statistical significance 5 days after exercise (P <
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Fig. 3. Transmission electron micrograph of muscle biopsy obtained 45 min postexercise (x24,000). Note the vast areas of Z-band streaming in this micrograph.

0.03). Expressed as the number of pixels above background (non-immune serum staining), IL-1\(\beta\) staining intensity increased from 1,613 ± 359 pixels/mm\(^2\) before exercise to 3,926 ± 772 pixels/mm\(^2\) 45 min after and to 5,904 ± 1,882 pixels/mm\(^2\) 5 days after and was localized to the pericellular space (Fig. 5). Muscle IL-1\(\beta\) levels were positively correlated to neutrophil infiltration (\(r = 0.687, P < 0.06\)). However, muscle IL-1\(\beta\) was not correlated to plasma levels of IL-1\(\beta\) after exercise, nor was it related to peripheral blood mononuclear cell production of IL-1\(\beta\) (previously reported in Ref. 5).

Creatine kinase. The pattern of creatine kinase response in these subjects has been reported previously (6). Briefly, creatine kinase levels rose in the young subjects after exercise from 81.9 ± 12.5 IU·l\(^{-1}\)·g creatinine\(^{-1}\), peaking at 307.2 ± 67.4 IU·l\(^{-1}\)·g creatinine\(^{-1}\) 24 h after exercise. In the present analysis, we observed a positive correlation between muscle IL-1\(\beta\) and plasma levels of creatine kinase when we combined values from before, immediately after, and 5 days after exercise (\(r = 0.416, P < 0.04\)). There was no relationship between creatine kinase levels and the percentage of Z-band damage.

DISCUSSION

The primary finding in the present study was the observed relationship between intramuscular immune parameters (neutrophil infiltration and IL-1\(\beta\) staining) and indexes of ultrastructural Z-band damage. These studies extend our previous systemic studies of inflammatory cells in exercise-induced skeletal muscle damage by using measurements made directly on muscle biopsy specimens.

A significant accumulation of intramuscular neutrophils was observed in biopsies obtained 45 min after the downhill running protocol; the level remained elevated 5 days after the exercise. Costill et al. (8) have shown increases in leukocytes in transverse muscle sections obtained 24 h after one-legged eccentric exercise. Recently, Widrick et al. (24) also reported increases in mononuclear cells between 6 and 72 h after one-legged eccentric exercise. Despite having an earlier and sustained time course, our own observations of increased neutrophil infiltration 45 min after exercise agree with the quantitative changes in circulating neutrophils after exercise in these same subjects (6). In that study neutrophil counts doubled with peak values occurring 6 h after exercise. The early rise and fall in circulating neutrophils suggests a rapid infiltration of these cells into muscle. The lower muscle neutrophil ratings 5 days postexercise compared with 45 min after exercise agree with the evidence that neutrophils live ~1-2 days after migrating into tissues (2).

Several studies have reported qualitative changes in muscle ultrastructure after eccentric exercise (15, 19, 23). However, few studies have attempted to quantify the magnitude of this muscle damage. We examined the volume of space occupied by damaged and undamaged Z-bands and focal damage in electron micrographs using a
Fig. 4. Transmission electron micrograph of muscle biopsy obtained from a subject 45 min postexercise (×24,000). Note the near-normal appearance with some areas of focal damage (FD).

quantitative test grid. The increased ratio of damaged to undamaged Z-bands in the immediate postexercise biopsies indicates that exercise-induced muscle injury occurs during exercise or early on in the postexercise recovery period. In the present study, measures of focal damage followed a similar pattern, showing strong positive correlation with Z-band damage. It is likely that the mechanical forces imposed by the eccentric contractions cause the initial damage observed 45 min after exercise. Disrupted muscle fiber fragments and released intracellular material may then form a chemotactic gradient that attracts neutrophils. Invading neutrophils may contribute to delayed metabolic responses in muscle after exercise. For example, Costill et al. (8) have suggested that the impaired muscle glycogen resynthesis observed after eccentric exercise may be due to competition between inflammatory cells and muscle cells for available glucose. Furthermore, the phagocytic and proteolytic activities of neutrophils may contribute to the clearance of damaged ultrastructural components. Recently, Zerba et al. (27) reported that the reduction in maximum isometric tetanic force after lengthening contractions was attenuated in animals injected with superoxide dismutase (a free radical scavenger), suggesting a causal role for reactive oxygen radicals in the etiology of eccentric exercise-induced ultrastructural damage.

Fig. 5. Plot of muscle IL-1β expressed as the number of pixels above non-immune staining background using non-immune rabbit serum/mm². *Significantly increased above pre-exercise (P < 0.03; n = 9).

Previous studies have noted increased plasma levels of IL-1β 3 to 6 h after exercise (4, 11). Previously, we reported that muscle levels of IL-1β increase immediately after exercise and remained elevated for up to 5 days after 45 min of downhill treadmill running. In the present study the use of quantitative image analysis techniques has enabled us to quantify changes in IL-1β staining intensity. We observed an increase in IL-1β staining intensity that was significantly elevated above baseline by 5 days after exercise. This sustained increase is consistent with the concept that IL-1β may contribute to the prolonged metabolic alterations after eccentric exercise. Prior studies have shown that eccentric exercise induces
myofibrillar protein breakdown for up to 10 days after a single bout of eccentric exercise (11, 13). IL-1β has been shown in conjunction with tumor necrosis factor to be a potent stimulator of muscle proteolysis in vivo (14, 26). Although dietary vitamin E supplementation had no effect on the intramuscular IL-1β levels reported here, vitamin E did inhibit a postexercise increase in IL-1β production measured in vitro in cells from these same subjects (5). This apparent discrepancy may be reconciled by noting that the rapid increase in intramuscular IL-1β (45 min) is not consistent with the time course of de novo synthesis of IL-1β (2–4 h). Therefore the IL-1β observed in muscle immediately after exercise may have been performed (in the lymph, for example). In the previous report (5), we found that vitamin E only influenced post-exercise IL-1β production, not basal (pre-exercise) production.

The relationship between muscle IL-1β staining and neutrophil infiltration is consistent with the role of this cytokine in directing leukocyte mobility. Previous studies have shown that IL-1β promotes endothelial adhesion and indirectly promotes chemotaxis of neutrophils by inducing production of IL-8 (7, 25). Also, blocking of IL-1β activity with IL-1β receptor antagonist has been shown to reduce IL-8 production by 85% in isolated peripheral blood mononuclear cells stimulated with IL-1β (20). The positive relationship between muscle IL-1β and creatine kinase may indicate the indirect influence of IL-1β via IL-8 on neutrophil degranulation and superoxide release that subsequently affects muscle membrane permeability.

In the present study we were unable to show any relationship between quantitative ultrastructural damage and plasma creatine kinase activity. These results agree with our previous observations and those of others suggesting that plasma creatine kinase activity is not a reliable index of ultrastructural damage (17, 22). It may, in fact, be a reflection of the metabolic adaptation to exercise-induced muscle damage.

In summary, the results of this study indicate that after eccentric exercise, neutrophils infiltrate skeletal muscle. Intracellular Z-band damage and the accumulation of IL-1β may induce neutrophil chemotaxis and adhesion. The resultant postexercise neutrophil influx may help clear damaged tissues in preparation for repair and hypertrophy.

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REFERENCES


