

# Protease Supplementation Improves Muscle Function after Eccentric Exercise

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## ABSTRACT

BUFORD, T. W., M. B. COOKE, L. L. REDD, G. M. HUDSON, B. D. SHELMADINE, and D. S. WILLOUGHBY. Protease Supplementation Improves Muscle Function after Eccentric Exercise. *Med. Sci. Sports Exerc.*, Vol. 41, No. 10, pp. 1908–1914, 2009. Protease supplementation has been purported to reduce the damaging effects of eccentric exercise and accelerate recovery of muscle function, possibly by regulating inflammation. **Purpose:** To determine the effectiveness of protease supplementation in attenuating eccentric exercise-induced skeletal muscle damage and inflammation. **Methods:** After standard physical and hemodynamic assessment and fasting venous blood samples, subjects performed isokinetic extension/flexion of the quadriceps group on a Biodex isokinetic dynamometer at  $60^{\circ}\cdot\text{s}^{-1}$ , followed by  $\dot{V}O_{2\text{max}}$  testing. Subjects were randomly assigned to consume 5.83 g daily of either a cellulose placebo ( $N = 15$ ;  $22.27 \pm 3.33$  yr,  $71.17 \pm 2.91$  inches,  $179.4 \pm 24.05$  lb,  $50.55 \pm 5.66$   $\text{mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ ) or a proteolytic supplement containing fungal proteases, bromelain, and papain ( $N = 14$ ;  $22.85 \pm 5.9$  yr,  $70.0 \pm 2.67$  inches,  $173.11 \pm 29.94$  lb,  $49.69 \pm 6.15$   $\text{mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ ) for a period of 21 d. After the supplementation period, subjects donated blood samples before performing a 45-min downhill ( $-17.5\%$ ) treadmill protocol at 60% of  $\dot{V}O_{2\text{max}}$ . An additional four blood draws and three muscle function tests were performed during the next 48 h. Blood was analyzed using standard hematology and clinical chemistry, enzyme-linked immunosorbent assay, and bead array. Blood data were analyzed using multivariate analysis of variance (MANOVA) with repeated measures, whereas Biodex data were analyzed using a MANOVA on  $\% \Delta$  values. **Results:** Significant group differences (T1–T3,  $P = 0.033$ ; T1–T4,  $P = 0.043$ ) and another strong trend (T1–3 h,  $P = 0.055$ ) were observed for flexion (peak torque  $\% \Delta$  at  $60^{\circ}\cdot\text{s}^{-1}$ ) indicating higher force production in the protease group. Significant group  $\times$  time interactions ( $P < 0.05$ ) were observed, including elevations in circulating eosinophils and basophils in the protease group coinciding with lower levels of serum cyclooxygenase 2, interleukin 6, and interleukin 12 in this group. **Conclusions:** Protease supplementation seems to attenuate muscle strength losses after eccentric exercise by regulating leukocyte activity and inflammation. **Key Words:** INFLAMMATION, MUSCLE DAMAGE, CYTOKINES, LEUKOCYTES, PROTEASES

Eccentric exercise involving lengthening muscle contractions have long been known to induce muscle damage by disruption of the sarcolemma (10,16,24). These events led to decreases in force production (11,12) and increases in delayed-onset muscle soreness (DOMS) that typically peak 2–3 d after the muscle injury (11). These disruptions to the sarcolemma present as ultrastructural damage, such as Z-line streaming (16), and as myofibrillar leakage of proteins, such as creatine kinase (CK), lactate dehydrogenase, myoglobin, myosin heavy chain, and troponin I (4,32). Integrating aerobic exercise with an eccentric component (i.e., downhill running) provides a proteolytic

challenge to muscle as it induces increases in serum cortisol (21), systemic oxidative stress (28), and sarcolemmal disruption (13). Evidence consistently shows that participants experience significant increases in CK and DOMS, with concomitant decreases in maximal isokinetic force after a 30- to 45-min bout of downhill running (13,21,28).

In addition to the mechanically induced damage, an inflammatory response to the muscle damage is also typically mounted by the immune system and is generally induced by elevated concentrations of circulating leukocytes (7,27), although opposing evidence does exist (22). Muscle inflammation is a poorly understood response to muscle damage that may occur after exercise, strain injury, or return to musculoskeletal loading after periods of inactivity (3,30,33). At present, it is unclear whether muscle inflammation is fundamentally harmful or beneficial to the muscle. Although evidence indicates that migration of inflammatory cells is an obligatory component of the muscle repair process (17,29,33), other data indicate that muscle force decrements after exercise cannot be fully explained by mechanical damage and are correlated with inflammatory cell invasion (14,19). Much interest in developing practical interventions to reduce muscle inflammation after muscle

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damage have been shown in the literature, as evidenced by numerous studies investigating the effects of nonsteroidal anti-inflammatory drugs such as cyclooxygenase 2 (COX2) inhibitors on muscle recovery from injury (26,35,39).

Another intervention that has shown promise in enhancing muscle recovery after damage is the oral ingestion of proteases. Proteases are a group of biologically active enzymes that are responsible for initiating protein catabolism via hydrolysis of peptide bonds that link amino acids together in a polypeptide chain. On the basis of their function and catalytic site of action, four primary groups of proteases can be delineated: serine proteases, cysteine proteases, aspartic acid proteases, and metalloproteases. Members of these groups can act as either exopeptidases responsible for cleavage of terminal amino acids (i.e., aminopeptidases or carboxypeptidases) or as endopeptidases responsible for attacking internal peptide bonds (i.e., trypsin, chymotrypsin, pepsin, papain, etc.). These enzymes are critically involved in numerous physiological processes ranging from the digestion of food particles to highly regulated systems such as blood clotting or immunological function.

Previous evidence has indicated that dietary supplementation with oral proteases may attenuate losses in skeletal muscle force production as well as muscle soreness after a damaging eccentric exercise (6,23). However, the mechanisms that underlie these effects remain largely a mystery. No role for the endogenous skeletal muscle proteases has been proposed; rather, these groups have hypothesized that reductions in muscle damage, DOMS, and/or force decrements were likely achieved via regulation of the well-characterized inflammatory process after eccentric exercise. Although these groups did not report mechanistic data, these hypotheses were primarily on the basis of classic observations of protease function. Two main functions of proteases were proposed to regulate the postexercise inflammatory process: 1) a reduction in the biosynthesis of eicosanoids via inhibition of the arachidonic acid cascade (36,40) and 2) a reduction in edema by improved mobilization of inflammatory cells from the tissues (9,31).

We hypothesized that oral protease ingestion would attenuate muscle damage and improve skeletal muscle function by reducing circulating macrophages and neutrophils after eccentric exercise and by regulating the effects of potent proinflammatory mediators such as tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) and interleukin  $1\beta$  (IL- $1\beta$ ). Therefore, the primary purposes of the present study were 1) to determine whether oral protease supplementation would improve skeletal muscle function after a 45-min downhill treadmill run and 2) to determine the potential effects that oral protease supplementation may have on the inflammatory response after muscle damage.

## MATERIALS AND METHODS

**Participants.** The present study used 29 recreationally active (consistent, structured exercise at least three times

per week) male subjects with a mean (SD) age of 22.55 (4.42) yr, height of 70.6 (2.81) inches, and body weight of 176.37 (26.75) lb. A total of 32 participants were recruited; however, 3 subjects voluntarily withdrew from the study. Only one participant withdrew because of symptoms thought to be attributable to the supplement. Participants with contraindications to exercise as outlined by the American College of Sports Medicine (ACSM) were not allowed to participate. All eligible participants signed university-approved informed consent documents, and approval was granted by the institutional review board for human subjects. In addition, all experimental procedures involved in the study conformed to the ethical consideration of the Helsinki Declaration. Investigators explained the purpose of the study, the protocol to be followed, and the experimental procedures to be used before allowing prospective participants to enter the study. After baseline testing, subjects were randomly assigned in a double-blind fashion to ingest either a carbohydrate placebo ( $N = 15$ ;  $22.27 \pm 3.33$  yr,  $71.17 \pm 2.91$  inches,  $179.4 \pm 24.05$  lb,  $50.55 \pm 5.66$  mL $\cdot$ kg $^{-1}\cdot$ min $^{-1}$ ) or a protease supplement ( $N = 14$ ;  $22.85 \pm 5.9$  yr,  $70.0 \pm 2.67$  inches,  $173.11 \pm 29.94$  lb,  $49.69 \pm 6.15$  mL $\cdot$ kg $^{-1}\cdot$ min $^{-1}$ ) for a period of 21 d.

**Experimental design.** The study was conducted in a randomized, double-blinded, and placebo-controlled manner. All subjects eligible to participate in the study completed a familiarization session where they were provided information (both verbal and written) regarding the study design, testing, and supplementation protocols. Informed consent, medical documents, and training history questionnaires were also completed at this time. Participants were then familiarized with the procedures to be encountered during the study. At this time, participants were given a verbal description of the downhill treadmill protocol but did not practice the exercise. At the completion of the familiarization session, participants were scheduled to return to the laboratory for baseline testing. Each participant was instructed to fast for 12 h and not to perform any physical activity for the 48 h preceding each testing session.

At baseline testing, participants completed isokinetic testing before performing a graded exercise test. After baseline measurements, participants were randomly assigned to either a placebo or a protease group. Participants were then instructed to return to the laboratory after 21 d of supplementation. Upon returning to the laboratory (T2), participants completed the eccentric exercise testing protocol. Isokinetic testing was then performed at 3, 24, and 48 h after exercise. Participants continued taking the supplement through the final day of testing.

**Aerobic capacity assessment.** At baseline, participants performed a volitional maximal cardiopulmonary exercise test according to the Bruce protocol (15). Participants were instructed to perform the test for as long as possible to ensure a true maximal attempt. Standard ACSM test termination criteria were monitored and followed throughout each test (1). Metabolic gases were

obtained with the Parvo Medics 2400 TrueMax metabolic measurement system (Sandy, UT) on a Trackmaster TMX425C treadmill (Newton, KS). The mean coefficient of variation (assessing maximum oxygen consumption) for this protocol has previously been shown to be 6.5% (range, 2%–14%) (15).

**Isokinetic muscular assessment.** At baseline and at 3, 24, and 48 h after the exercise bout, participants performed isokinetic muscle testing on a Biodex Isokinetic Dynamometer (Biodex Medical Systems, Shirley, NY) on their dominant leg. Before testing, participants performed a standard warm-up by pedaling at 50–60 rpm on a Monark cycle ergometer (Varberg, Sweden). After performing warm-up repetitions before each set, participants performed a total of three sets of five repetitions of quadriceps extension/flexion on each leg at a speed of  $60^{\circ}\cdot\text{s}^{-1}$ .

**Eccentric exercise bout.** After 21 d of supplementation, participants performed an eccentrically biased aerobic exercise test on the basis of previous guidelines (18,23,28). On a treadmill positioned downhill at a grade of  $10^{\circ}$  ( $-17.6\%$ ), participants ran for 45 min at 60% of their  $\dot{V}O_{2\text{max}}$ . Oxygen consumption was monitored every 5 min, and the treadmill speed was adjusted accordingly to maintain intensity at  $\sim 60\%$  of  $\dot{V}O_{2\text{max}}$ . Water was given *ad libitum* during the exercise.

**Venous blood sampling and muscle biopsies.** Subjects were required to fast for 12 h before donating approximately 20 mL of venous blood from an antecubital vein using standard phlebotomy procedures. A total of six blood samples were donated during the study. Blood analyzed for cytokines, markers of oxidative stress, and markers of the arachidonic acid pathway were placed into two serum separation tubes and immediately centrifuged at 1100g for 15 min. Serum was separated and stored at  $-80^{\circ}\text{C}$  in polypropylene tubes for later analysis. Blood to be analyzed for circulating leukocytes was collected in a single lavender top tube containing  $\text{K}_2$  EDTA and refrigerated for approximately 1–3 h for subsequent analysis using an Abbott Cell Dyn 3500 (Abbott Laboratories, Abbott Park, IL) automated hematology analyzer.

**Dietary records.** The subjects' diets were not standardized, and subjects were asked not to change their dietary habits during the study. However, subjects were required to keep a record of all dietary consumption for the 21-d supplementation period. The dietary records were evaluated with the Food Processor dietary assessment software program (ESHA Research, Salem, OR) to determine the average daily macronutrient consumption as well as the intake of vitamins C and E in the diet.

**Perceived muscle soreness.** As a subjective indicator of the severity of muscle injury, perceived soreness was assessed along a 13-cm scale (0 = no soreness, 13 = extreme soreness). Participants rated their level of soreness before and 3, 24, and 48 h after exercise by drawing an intersecting line across the continuum line extending from 0 to 13. The distance of each mark was measured from

0, and the measurement was used as the perceived level of soreness.

**Supplementation protocol.** Subjects were assigned to ingest in a double-blind randomized manner 5.828 g daily of either a cellulose placebo ( $N = 15$ ) or a protease supplement ( $N = 14$ ) for a period of 24 d. Supplements were ingested for 21 d before returning to the laboratory for testing and during the 3 d of data collection. The protease supplement consisted of 47.7 mg of papain, 99.9 mg of bromelain, 5.593 g of fungal enzymes with endopeptidase/exopeptidase activity, and 86.4 mg of calcium citrate. Supplements were packaged in identical capsules, and subject compliance was monitored by completion of a log of capsules consumed per day.

**Serum analyses.** Serum levels of CK, immunoglobulin (Ig) G, IgA, and IgM were assessed using a DADE Dimension RXL clinical chemistry analyzer (Dade-Dehring, Inc, Newark, DE). The analyzer was calibrated daily using Liquid Assayed Multiqual Control (Bio-Rad, Hercules, CA), and two levels of quality control with known concentrations were performed.

Serum levels of IL-6, IL-8, IL-10, IL-12, TNF- $\alpha$ , and IL-1 $\beta$  were analyzed using the Bio-Plex Bead Array Analysis System (Bio-Rad). Bio-Plex assays contain dyed beads conjugated with monoclonal antibodies specific for target proteins or peptides such as cytokines or phosphoproteins. Each of the addressed bead sets can contain a capture antibody specific for a unique target protein. The antibody-conjugated beads are allowed to react with a sample and a secondary (to detect) antibody in a microplate well to form a capture sandwich immunoassay. Multiplex assays can be created by mixing bead sets with different conjugated antibodies to simultaneously test for many analytes in one sample. The Luminex 100 instrument (Austin, TX) was used per manufacturer's protocol for data acquisition, and standard curves were constructed using accompanying statistical software. These standard curves were generated for all measures using commercially developed standards with manufacturer-reported linearity of  $>0.995$  and accuracy of  $>90\%$  at  $<200$  MESF. Manufacturer-recommended intra-assay coefficients of variation are 5%–10%; assays run in this study resulted in a mean coefficient of variation of 8.46%.

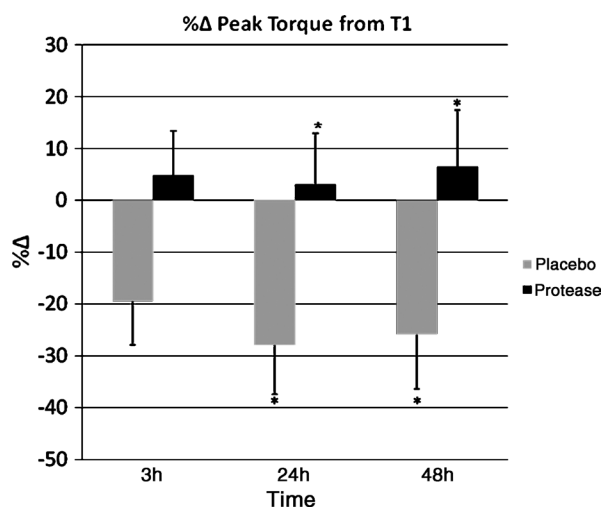
Serum levels of superoxide dismutase (SOD), prostaglandin  $\text{E}_2$  ( $\text{PGE}_2$ ), and 8-isoprostane (8-iso) were determined using commercially available enzyme immunoassay (EIA) kits from Cayman Chemical, Co (Ann Arbor, MI). Serum levels of COX2 were determined using commercially available enzyme-linked immunosorbent assay (ELISA) kits from EMD Chemicals, Inc. (San Diego, CA). Concentrations of each target protein were determined using the colorimetric method at an optical density of either 450 (ELISA) or 405 nm (EIA) with a microplate reader (Wallac Victor 1420; Perkin Elmer, Boston, MA). Standard curves were generated using a commercially available microplate reader-compatible statistical software (MicroWin 2000, Microtek

Laborsysteme GmbH, Overath, Germany). These standard curves were generated for all measures using commercially developed standards with specific antigens with reported *r* values in the range of 0.948–0.999. Intra-assay coefficients of variation for each assay were determined for each duplicate for all participants and resulted in a mean coefficient of variation of 2.90%.

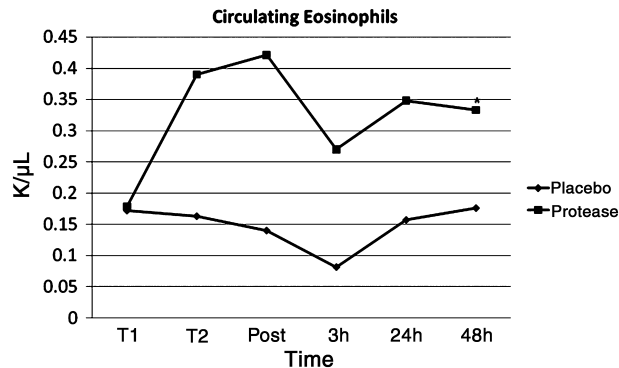
**Statistical analyses.** Blood data were analyzed using a 2 × 6 (group × blood sample) factorial multivariate analysis of variance (MANOVA) with repeated measures and univariate follow-up tests. To protect against a type I error, the conservative Hunyh–Feldt epsilon correction factor was used to evaluate the observed within-group *F* ratios when the sphericity assumption was not met. Isokinetic data were converted to delta scores (baseline-subtracted) and analyzed using separate MANOVA with univariate follow-up. Nutrition data were also analyzed using a MANOVA with univariate follow-up. Statistical analyses were performed using the SPSS 16.0 for Windows software package (Chicago, IL) and significance was set at *P* < 0.05 (two-tailed) throughout.

## RESULTS

**Force production and muscle soreness.** Force production was analyzed by using a Biodex isokinetic dynamometer to assess maximal peak torque production. These values were converted to a percent change value relative to the baseline value. Significant group differences were observed for the percent change in maximal peak torque at 60°·s<sup>-1</sup> (dominant leg flexion) from T1 at 24 (*P* = 0.033) and 48 h (*P* = 0.043) after exercise, whereas a strong trend (*P* = 0.055) was observed for the change from T1 to 3 h after exercise (Fig. 1). No significant differences were observed between groups for the extension



**FIGURE 1**—Percent changes in isokinetic peak torque at 60°·s<sup>-1</sup>. Data presented as mean ± SE in the protease and placebo groups 3 h after exercise (3h), 24 h after exercise (24h), and 48 h after exercise (48h). \*Significantly different between groups (*P* < 0.05).

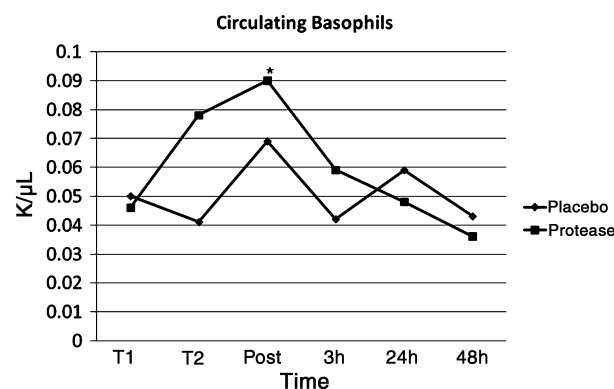


**FIGURE 2**—Time course of numbers of circulating eosinophils. Data presented as mean in the protease and placebo groups at baseline (T1), after supplementation/before exercise (T2), immediately after exercise (Post), 3 h after exercise (3h), 24 h after exercise (24h), and 48 h after exercise (48h). \*Significant (*P* = 0.045) increase in circulating eosinophils from T1 to 48 h after exercise in the protease group compared with the placebo group.

portion of the exercise or for subjective ratings of muscle soreness.

**Circulating leukocytes.** Venous blood samples were analyzed for circulating numbers of leukocytes. We observed significant time effects for neutrophils (*P* < 0.001), monocytes (*P* < 0.001), lymphocytes (*P* = 0.004), eosinophils (*P* = 0.032), and basophils (*P* = 0.007). Significant group × time interactions were observed for eosinophils (*P* = 0.045) and basophils (*P* = 0.015), whereas no significant interactions were observed for circulating neutrophils, monocytes, or lymphocytes. Data for circulating eosinophils and basophils are shown in Figures 2 and 3, respectively. Eosinophils were significantly elevated in the protease group after supplementation, whereas basophils increased to a greater degree in the protease group before falling below levels of the placebo group at 24 and 48 h after exercise.

**Serum immunoglobulins and CK.** Serum samples from the venous blood samples were analyzed for several



**FIGURE 3**—Time course of numbers of circulating basophils. Data presented as mean in the protease and placebo groups at baseline (T1), after supplementation/before exercise (T2), immediately after exercise (Post), 3 h after exercise (3h), 24 h after exercise (24h), and 48 h after exercise (48h). \*Significant (*P* = 0.015) increase in circulating basophils from T1 to immediately after exercise in the protease group compared with the placebo group.

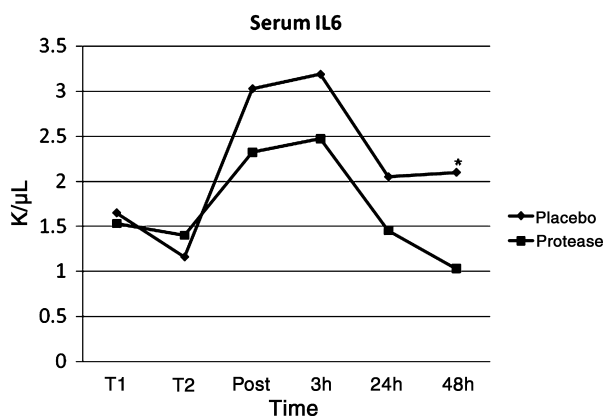
immunoglobulins and for CK. Significant time effects were observed for serum IgG ( $P < 0.001$ ) and IgA ( $P = 0.004$ ), whereas no significant changes were observed for serum IgM. No significant differences were observed between groups for any of the immunoglobulins. Serum CK was significantly ( $P < 0.001$ ) elevated in both groups in response to the exercise, demonstrating a significant level of muscle damage. However, the protease supplement did not attenuate serum CK compared with placebo.

#### Serum cytokines, SOD, PGE<sub>2</sub>, COX2, and 8-iso.

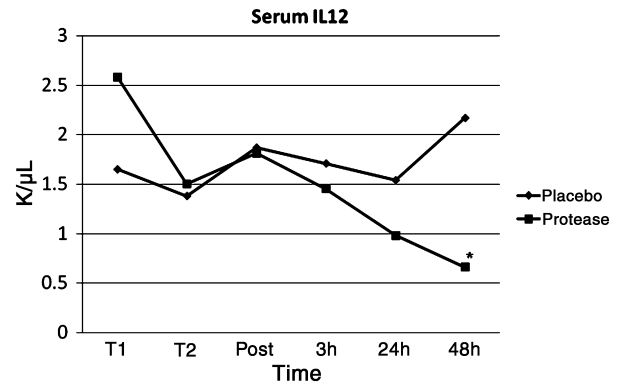
For serum cytokines, significant time effects were observed for IL-1 $\beta$  ( $P = 0.040$ ), IL-6 ( $P < 0.001$ ), IL-8 ( $P = 0.018$ ), IL-10 ( $P = 0.028$ ), and IL-12 ( $P = 0.029$ ), whereas no significant changes were observed in TNF- $\alpha$ . Significant group  $\times$  time interactions were observed for IL-6 ( $P = 0.035$ ) and IL-12 ( $P = 0.023$ ), whereas no other cytokines showed significant interactions between groups. Data for serum IL-6 and IL-12 are shown in Figures 4 and 5, respectively. Serum IL-6 was significantly higher after exercise in the placebo group, whereas the levels of IL-12 in the protease group dipped below those of the placebo group beginning at 3 h after exercise.

Significant time effects were observed for serum SOD activity ( $P < 0.001$ ), 8-iso ( $P < 0.001$ ), and COX2 ( $P = 0.041$ ), whereas no significant alterations were observed for PGE<sub>2</sub> or SOD. No significant interactions were observed for SOD, PGE<sub>2</sub>, or 8-iso. Meanwhile, a significant group  $\times$  time interaction was observed for COX2 ( $P = 0.005$ ). COX2 was elevated significantly more within the placebo group after the exercise with respect to the protease group. Meanwhile, a weak trend ( $P = 0.084$ ) for decreasing PGE<sub>2</sub> in the protease group was observed.

**Nutritional analyses.** No significant differences were observed for average daily ingestion of total calories, fat, protein, carbohydrates, vitamin E, or vitamin C between groups. Mean  $\pm$  SD daily nutritional intake for the placebo



**FIGURE 4**—Time course of serum levels of IL-6. Data presented as mean in the protease and placebo groups at baseline (T1), after supplementation/before exercise (T2), immediately after exercise (Post), 3 h after exercise (3h), 24 h after exercise (24h), and 48 h after exercise (48h). \*Significant ( $P = 0.035$ ) increase in serum IL-6 from T2 to 48 h after exercise in the placebo group compared with the protease group.



**FIGURE 5**—Time course of serum levels of IL-12. Data presented as mean in the protease and placebo groups at baseline (T1), after supplementation/before exercise (T2), immediately after exercise (Post), 3 h after exercise (3h), 24 h after exercise (24h), and 48 h after exercise (48h). \*Significant decrease ( $P = 0.023$ ) in serum IL-12 from immediately after exercise to 48 h after exercise in the protease group compared with the placebo group.

group was 2079.76  $\pm$  355.2 kcal, 91.25  $\pm$  16.16 g protein, 244.55  $\pm$  50.52 g carbohydrate, 83.61  $\pm$  19.15 g fat, 48.56  $\pm$  16.09 mg vitamin C, and 4.69  $\pm$  1.86 mg vitamin E. Mean  $\pm$  SD daily nutritional intake for the protease group was 2234.11  $\pm$  518.49 kcal, 101.05  $\pm$  25.43 g protein, 263.07  $\pm$  69.02 g carbohydrate, 87.61  $\pm$  24.10 g fat, 52.61  $\pm$  23.94 mg vitamin C, and 6.61  $\pm$  6.29 mg vitamin E.

## DISCUSSION

The results of the present study indicate that 21 d of dietary supplementation with orally ingested proteases significantly improves muscle function after an eccentrically damaging exercise. These results are in agreement with previous findings (6,23). In the present study, we used a downhill running protocol similar to that used by Miller et al. (23) in physically active males between the ages of 18 and 35 yr. As expected, the placebo group showed significant drops in force production as indicated by the maximal torque production of the quadriceps muscles during flexion movements. Interestingly, the protease group showed similar decrements in force production during the extension movements but showed no force decrements in the flexion portion of the exercise and actually increased force production from baseline at 3, 24, and 48 h after exercise. In both groups, significant levels of serum CK were observed, indicating a significant level of muscle damage in each group. The CK data support previous reports that the beneficial effects of protease supplementation are likely due to the differences in the inflammatory muscle recovery process rather than the prevention of contraction-induced muscle damage.

It has been proposed that potential benefits of protease supplements on muscle function are via interactions with the immune system through the regulation of inflammation. One of the primarily proposed mechanisms involves the regulation of cellular inflammation. The primary immune

cells known to regulate the postexercise inflammatory response are macrophages and neutrophils. These cells are responsible for clearing cellular debris by infiltration into damaged tissue by passing through the interstitium after vascular dilation and swelling. Much remains unknown about the function of neutrophils and macrophages in the muscle repair process because they are each known to be able to induce additional muscle damage or muscle repair in varied situations (34). In fact, it has been observed that phagocytic cells can actually change from pro- to anti-inflammatory several days after injury (5). We did not observe any significant differences between groups for circulating numbers of either neutrophils or monocytes at any of our measured time points. In addition, no significant differences were observed for either TNF- $\alpha$  or IL-1 $\beta$ , both of which are powerful inducers of pro-inflammatory M1 macrophages. However, significantly greater serum levels of IL-6 and IL-12 were observed after exercise, each being a potent macrophage-released inflammatory mediator. It seems likely that reductions in circulating IL-6 and IL-12 in the protease group may have contributed reduced muscle inflammation and thus improved muscle force.

By far our most intriguing finding is the increase in circulating eosinophils and basophils in the protease group. These leukocytes are typically associated with allergic reactions involving mast cells and IgE. Mast cells, eosinophils, and basophils each typically contribute to increasing tissue inflammation by releasing cytokines and chemokines, which increase tissue permeability and allow extravasation of other leukocytes. Eosinophils and mast cells are often thought of in negative terms because they have potentially harmful toxic molecules that may be released when they degranulate. Mast cells are capable of releasing histamine, leukotrienes, and TNF- $\alpha$ , whereas eosinophils are capable of secreting different eicosanoids and triggering histamine release from mast cells. In the present study, we observed no significant differences between groups for either prostaglandin E<sub>2</sub> or TNF- $\alpha$ . In addition, only 2 of 16 individuals who took the protease reported perceived adverse effects from the supplement and only 1 withdrew from the study because of these perceived effects (another withdrew for unrelated reasons). We also observed significantly lower levels of COX2, the primary signal for the production of prostaglandins, in the protease group. This result not only indicates a potential beneficial effect on health but also seems to support the hypothesis that protease supplementation may reduce inflammation by inhibiting the arachidonic acid cascade. Although no significant differences were observed for the serum levels of PGE<sub>2</sub>, the observed trend ( $P = 0.084$ ) in conjunction with decreases in COX2 indicate a potential clinical benefit to the protease supplementation. It also remains a possibility that other prostaglandins may be altered and thus mediate the improvements in muscle function.

Mast cells and eosinophils also perform other overlooked functions such as providing chemotactic signals for phago-

cytes (e.g., IL-4) and signaling restructuring of the extracellular matrix (ECM) through activation of metalloproteases by release of enzymes such as tryptase, carboxypeptidase, and eosinophil collagenase. These functions led us to propose two hypotheses concerning the effects of proteases on muscle recovery. First, it is possible that the protease altered the recruitment of these phagocytic cells to the muscle via alterations in the involvement of chemokines and/or chemokine receptors. These molecules are known to contribute to the muscle regeneration response after injury by recruitment of macrophages and/or neutrophils to the site of injury (29,37,38). Secondly, ECM remodeling seems to be a possible explanation for the protease-mediated improvements in muscle function. The ECM primarily contributes to force transmission by providing structural support for skeletal muscle. It has been reported that eccentric exercise contributes significantly to the remodeling of ECM components (20). Remodeling of the ECM is thought to be responsive to mechanical stress (8), but the matrix metalloproteases (MMP) are the primary agents responsible for the breakdown of ECM components. MMP are zinc-dependent proteases that are secreted as zymogens and are activated by enzymatic cleavage of the propeptide. Not only do these proteases regulate collagen breakdown but also they are thought to increase myoblast migration across the basement membrane and connective tissue barriers to reach the site of muscle injury during regeneration (2,25). Although downhill running has been shown to activate MMP, it seems plausible that the MMP were further activated either by the ingested proteases directly or by the release of mast cell proteases and thus contributed to improved muscle function after the downhill run. Future studies investigating the relationship among protease supplementation, chemokines, and MMP seem warranted.

In conclusion, we observed significant improvements in muscle function for 48 h after a 45-min downhill treadmill run in a protease-supplemented group when compared with placebo. It seems that these improvements were due to the alterations in the systemic inflammatory response to muscle damage rather than to a reduction in damage itself. Our data indicate that macrophage-derived inflammatory mediators were reduced in the protease group after the exercise protocol. Our data also led us to speculate that it remains possible that inhibition of the arachidonic acid pathway plays a role because of the significant repression of COX2 in the protease group. Finally, it seems quite possible that the positive effects of the proteases on muscle function are due to the activation of MMP that contribute to ECM remodeling and myoblast migration during periods of muscle regeneration.

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