

# Acute phase response in exercise. II. Associations between vitamin E, cytokines, and muscle proteolysis

JOSEPH G. CANNON, SIMIN N. MEYDANI, ROGER A. FIELDING,  
MARIA A. FIATARONE, MOHSEN MEYDANI, MEHRDAD FARHANGMEHR,  
SCOTT F. ORENCOLE, JEFFREY B. BLUMBERG, AND WILLIAM J. EVANS  
*Human Physiology and Nutritional Immunology and Toxicology Laboratories, United States  
Department of Agriculture Human Nutrition Research Center on Aging at Tufts University,  
and Department of Medicine, Tufts University School of Medicine  
and New England Medical Center, Boston, Massachusetts 02111*

CANNON, JOSEPH G., SIMIN N. MEYDANI, ROGER A. FIELDING, MARIA A. FIATARONE, MOHSEN MEYDANI, MEHRDAD FARHANGMEHR, SCOTT F. ORENCOLE, JEFFREY B. BLUMBERG, AND WILLIAM J. EVANS. *Acute phase response in exercise. II. Associations between vitamin E, cytokines, and muscle proteolysis*. Am. J. Physiol. 260 (Regulatory Integrative Comp. Physiol. 29): R1235–R1240, 1991.—Cytokines such as interleukin 1 (IL-1), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and interleukin 6 (IL-6) mediate a variety of host responses to trauma and infection, including skeletal muscle proteolysis. This investigation assesses the influence of damaging eccentric exercise on in vitro production and plasma concentrations of cytokines and their relationship to muscle protein breakdown. In a double-blind placebo-controlled protocol, 21 male subjects took vitamin E supplements (800 IU/day) for 48 days, then ran downhill on an inclined treadmill. Twenty-four hours after this single session of eccentric exercise, endotoxin-induced secretion of IL-1 $\beta$  was augmented 154% ( $P < 0.01$ ) in cells obtained from the placebo subjects, but no significant exercise-related changes were observed in cells from the vitamin E-supplemented subjects. TNF- $\alpha$  secretion was also significantly increased 24 h after exercise, but the response was not inhibited by vitamin E. In contrast, IL-6 secretion did not change after exercise, but dietary vitamin E supplementation significantly reduced IL-6 secretion throughout the 12-day period of observation ( $P = 0.023$ ). Urinary 3-methylhistidine excretion correlated with mononuclear cell secretion of both IL-1 $\beta$  ( $P < 0.05$ ) and prostaglandin E<sub>2</sub> ( $P < 0.05$ ), supporting the concept that these mononuclear cell products contribute to the regulation of muscle proteolysis.

interleukin 1; tumor necrosis factor- $\alpha$ ; interleukin 6; 3-methylhistidine

LONG-DURATION or damaging exercise often initiates reactions that resemble the “acute phase response” to infection (5). These include mobilization and activation of neutrophils (6), proteolysis of skeletal muscle (12), and increased hepatic production of certain plasma proteins (21). During infection, the reactions are thought to be functionally related: proteolysis of skeletal muscle mobilizes amino acids for hepatic synthesis of antioxidant enzymes and antiproteases. These proteins, in turn, limit damage to the host from the antimicrobial oxygen radicals and proteases released by neutrophils. These

various elements of the acute phase response are unified by a common set of mediators (cytokines), including interleukin 1 $\beta$  (IL-1 $\beta$ ), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and interleukin 6 (IL-6) (11). Cytokines influence some of their target tissues via induction of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), which also serves as a negative-feedback inhibitor of cytokine production (20).

We have hypothesized that damaging exercise initiates an acute phase response that contributes to the breakdown and clearance of overloaded tissue. After eccentric exercise, a particularly damaging activity involving forced lengthening of a muscle as it develops tension (1), we found a significant relationship between neutrophil function and muscle membrane permeability (6). Moreover, the results were significantly influenced by the age of the subjects and by dietary intake of vitamin E. The present report examines the changes in mononuclear cells, PGE<sub>2</sub>, and cytokines in this same population of subjects and the possible association of these factors with muscle catabolism, as assessed by urinary excretion of 3-methylhistidine, a nonrecycled breakdown product of myofibrillar protein (12).

## METHODS

**Human subjects protocol.** Sedentary men representing two ranges of age [ $25 \pm 3$  (SD) yr,  $n = 9$ ; and  $65 \pm 2$  yr,  $n = 12$ ], with normal weight for their height, were enrolled in the study. Before acceptance in the study, all subjects passed a complete physical exam and completed a maximal O<sub>2</sub> uptake ( $\dot{V}O_{2\max}$ ) test on a cycle ergometer. Using a double-blind random-allocation system, four <30-yr-old and six >55-yr-old subjects supplemented their diet with *dl*- $\alpha$ -tocopherol (vitamin E) taken as capsules (400 IU in soybean oil, twice daily, Hoffmann-La Roche, Nutley, NJ). The supplements were taken for 48 days before exercise to allow incorporation into membrane lipids. The other subjects ingested placebo (containing only soybean oil with a total  $\alpha + \gamma$ -tocopherol content of <0.5 IU) for the same period of time. The supplements were discontinued on the day of exercise and for 2 days after to investigate the influence of the exercise on plasma vitamin E levels without complications arising from intestinal absorption, then continued

for the remainder of the study. The exercise session consisted of three 15-min periods of downhill running on a treadmill inclined  $-16\%$ . The periods were separated by 5 min of rest. The exercise intensity was set at  $75\%$  of each subject's maximum heart rate, as determined by the  $\dot{V}O_{2\max}$  test performed several months before. Blood samples were obtained before, immediately after, 3 and 6 h after, and 1, 2, 5, and 12 days after exercise. Complete 24-h urine collections were obtained the day before exercise, the day of exercise, and 1, 2, 5, and 12 days after exercise. The subjects ate a self-selected meat-free diet for at least 3 days before each of the urine collection periods and were instructed to abstain from exercise, vitamin supplements, and all antiinflammatory or analgesic drugs throughout the course of the study. Rectal temperature was measured using a flexible thermistor probe (Yellow Springs Instrument, Yellow Springs, OH) before 8 A.M. on the day of exercise and on the day after exercise, and at 2-h intervals between 2 and 12 h post-exercise. Needle biopsies were taken from the vastus lateralis muscles of the  $<30$  yr subjects immediately before and after, and 5 days after the exercise for other studies outside the scope of this report. The protocol was approved by the Tufts University-New England Medical Center Human Investigation Review Committee.

**Hematology.** Complete blood counts were obtained using a Baker 9000 hematology analyzer (Serono-Baker Instrument, Allentown, PA). White cell differential counts were determined by microscopic examination of Wright-Giemsa stained blood smears.

**Urinary creatinine and 3-methylhistidine.** Creatinine analysis was carried out using a Cobas Fara II automated centrifugal analyzer and reagent kit (Roche Diagnostic Systems, Nutley, NJ). Urinary creatinine was reacted with picric acid, and the rate of red complex formation was measured at 520 nm. 3-Methylhistidine was isolated by using reverse-phase high-performance liquid chromatography (HPLC) and detected by fluorescence (34).

**Leukocyte isolation.** Plasma was separated from heparinized blood by centrifugation. The packed cells were resuspended in culture medium (RPMI) supplemented with L-glutamine (0.3 mg/ml), penicillin (100 U/ml), streptomycin (100  $\mu$ g/ml) (all from GIBCO, Grand Island, NY), 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Microbiological Associates), 2 mercaptoethanol (Eastman Kodak, Rochester, NY), and subjected to density-gradient centrifugation using ficoll-hypaque (Ficoll-Paque; Sigma, St. Louis, MO). The mononuclear cell layer was recovered and washed twice with supplemented RPMI.

**In vitro cytokine production and assay.** The mononuclear cells were suspended at a final concentration of  $2.5 \times 10^6$  cells/ml in supplemented RPMI with 2% heat-inactivated AB serum in 24-well plates (Becton Dickinson, Oxnard, CA). The cells were incubated in a humidified, 5%  $CO_2$  atmosphere at  $37^\circ C$  for 48 h with or without 1 ng/ml lipopolysaccharide (LPS) endotoxin (*Escherichia coli* 1335, Sigma). After incubation, the supernatants were aspirated and centrifuged at 10,000 *g* for 1 min to remove any aspirated cells, then stored frozen in polypropylene microcentrifuge tubes until evaluated by radioimmunoassay (RIA). IL-1 $\beta$ , TNF- $\alpha$ , and IL-6 con-

centrations were measured by competitive-inhibition RIAs using  $^{125}I$ -labeled tracers and polyclonal rabbit antisera developed in collaboration with Dr. Charles Dinarello, Dept. of Medicine, New England Medical Center (7, 32).

**Plasma cytokine analyses.** Separate blood samples were collected in tubes containing EDTA (1.5 mg/ml of blood) and aprotinin [0.67 trypsin inhibitor units (TIU)/ml] on the day of exercise and the next morning. After centrifuging the blood at 400 *g* for 10 min, the plasma was drawn off and recentrifuged at 10,000 *g*. The resultant platelet-free plasma was stored at  $-70^\circ C$ . Plasma samples evaluated with the IL-1 $\beta$  RIA were first extracted with chloroform (7). The performance of the IL-1 $\beta$  and TNF- $\alpha$  RIAs with human plasma samples, including dilutional characteristics and cytokine recovery, have been reported in detail elsewhere (7). Although plasma IL-6 determinations were attempted with the IL-6 RIA and with an experimental enzyme-linked immunosorbent assay (ELISA) method, plasma-associated interference was encountered, and reliable results were not obtained.

**PGE<sub>2</sub> production and assay.** Mononuclear cells ( $10^6$  cells/ml) were cultured in 24-well plates with RPMI containing 10% fetal bovine serum (GIBCO) with or without 10  $\mu$ g/ml phytohemagglutinin (PHA-P, Difco, Detroit, MI) for 48 h in the ambient conditions described above. Cell-free supernatants were stored at  $-70^\circ C$  until analysis of PGE<sub>2</sub> by RIA (22). PGE<sub>2</sub> antibody was a gift from Drs. J. Dupont of Iowa State and M. Mathias of Colorado State Universities. The antibody cross-reactivity and specificity have been described (24). Leukotriene B<sub>4</sub> (LTB<sub>4</sub>) was measured using a commercial RIA kit (Advanced Magnetics, Cambridge, MA).

**Statistical analyses.** Values reported are means  $\pm$  SE. Differences compared to preexercise baseline were assessed by one-factor repeated-measures analysis of variance (ANOVA). The overall influences of age or dietary vitamin E supplementation were determined by two-factor repeated-measures ANOVA. Differences between groups at specific times were determined by factorial ANOVA. The ANOVAs were followed by Fisher's least significant difference test. Plasma cytokine determinations below the detection limit of the assay were assigned the value at the detection limit (IL-1 $\beta$ : 15 pg/ml, TNF- $\alpha$ : 10 pg/ml) for statistical analysis by the nonparametric Mann-Whitney *U* and Kruskal-Wallis tests. All analyses were performed on a Macintosh SE/30 computer with Statview SE software (Abacus Concepts, Berkeley, CA). Rank correlations are expressed as Spearman's rho ( $\rho$ ). Because of limitations of sample volumes and cell yields, not all assays were possible on all subjects at all times. The number of subjects sampled is specified in figure legends. No significant differences were attributable to the age of the subjects, therefore all data are grouped according to dietary supplement.

## RESULTS

**Cytokine secretion in vitro.** Before exercise, IL-1 $\beta$  and TNF- $\alpha$  secretion by mononuclear cells isolated from the vitamin E-supplemented subjects were similar to the

placebo subjects, but IL-6 secretion was approximately half ( $P = 0.059$ ) of the placebos. Within the first 6 h after exercise, no significant changes from baseline occurred in IL-1 $\beta$ , TNF- $\alpha$ , or IL-6 secretion (data not shown). At 24 h, IL-1 $\beta$  secretion by cells from placebo subjects increased 154% over baseline ( $P < 0.05$ , Fig. 1). In contrast, cells from the vitamin E-supplemented subjects exhibited no significant increase in IL-1 $\beta$  secretion after exercise. At 2 and 5 days after exercise in vitro, IL-1 $\beta$  secretion for several of the vitamin E-supplemented subjects was less than preexercise baseline.

Mononuclear cells from subjects taking vitamin E secreted significantly less IL-6 than cells from placebo subjects throughout the 12-day period of observation ( $P = 0.023$ ). TNF- $\alpha$  secretion, on the other hand, exhibited no significant differences due to vitamin E supplementation. Although TNF- $\alpha$  and IL-6 secretion were both increased somewhat the day after exercise (~60 and 40%, respectively), only the increase in TNF- $\alpha$  was significant ( $P < 0.05$ ). The secretion of each of the three cytokines was independent, i.e., cells that were high producers of one cytokine were not necessarily high producers of others.

The changes in cytokine secretory capacity were not associated with changes in mononuclear cell numbers. There were no significant exercise-induced changes, or vitamin E-related differences, in circulating monocyte or lymphocyte counts.

Cells from the vitamin E-supplemented subjects produced ~260% more PGE<sub>2</sub> throughout the 12 days of observation, but because of the large degree of variability, the difference was not significant ( $P = 0.154$ , Fig. 2). PGE<sub>2</sub> secretion was significantly increased after exercise in cells from the placebo subjects but not the vitamin E-supplemented subjects.

**Urinary 3-methylhistidine.** Urinary excretion of 3-methylhistidine was variable (Fig. 3). The overall mean was greatest at 12 days after exercise, but the values were less than preexercise baseline for several of the vitamin E-supplemented subjects, particularly 2 and 5 days after exercise. No significant vitamin E-related differences between groups were observed. Nevertheless, a significant longitudinal relationship ( $\rho = 0.257$ ,  $P < 0.05$ ) was observed over time between the change in 3-methylhistidine excretion and the change in IL-1 $\beta$  secretion, relative to preexercise baseline. Figure 4A shows the data from six subjects that responded with the greatest changes in 3-methylhistidine (>35% increase or decrease from baseline). These subjects exhibited a somewhat

stronger correlation between changes in 3-methylhistidine and IL-1 $\beta$  ( $\rho = 0.418$ ,  $P < 0.05$ ). In addition, a cross-sectional correlation of 3-methylhistidine excretion with in vitro secretion of both IL-1 $\beta$  ( $\rho = 0.479$ ,  $P < 0.05$ , Fig. 4B) and PGE<sub>2</sub> ( $\rho = 0.742$ ,  $P < 0.001$ ) was observed 12 days after exercise for all subjects. 3-Methylhistidine excretion did not correlate with TNF- $\alpha$  or IL-6.

**Plasma IL-1 $\beta$  and TNF- $\alpha$ .** Except at 6 h after exercise (Fig. 5), the plasma cytokine concentrations were distributed near the detection limits of the assays (Table 1). However, all of the plasma samples that did have IL-1 $\beta$  immunoreactivity were from placebo subjects (Fig. 5, Table 1). In contrast, immunoreactive TNF- $\alpha$  was found in plasma from both placebo and vitamin E groups (Table 1). Circadian variations in these cytokines have not been observed in previous studies (5).

## DISCUSSION

The results of this investigation indicate that cytokine secretion by LPS-stimulated mononuclear cells is significantly influenced by exercise and by dietary vitamin E supplementation. Furthermore, changes in muscle protein breakdown, as measured by urinary 3-methylhistidine excretion, were associated with changes in IL-1 $\beta$  and PGE<sub>2</sub> secretion from mononuclear cells.

**Cytokine secretion.** IL-1 $\beta$  and TNF- $\alpha$  secretion was significantly increased the morning after exercise without any concurrent changes in mononuclear cell numbers. In addition, vitamin E supplementation affected the secretion in a manner that was specific for each cytokine: IL-6 secretion was decreased overall; exercise-enhanced IL-1 $\beta$  secretion was suppressed, but baseline IL-1 $\beta$  secretion was not affected; and TNF- $\alpha$  secretion was not affected at all. Several mechanisms can be postulated to account for these alterations in cytokine secretion, and in view of the differential alterations observed, different mechanisms or combinations of mechanisms may govern each cytokine.

Vitamin E influences arachidonic acid metabolism, and both cyclooxygenase and lipoxygenase pathways, in turn, influence cytokine production (20). PGE<sub>2</sub> is an inhibitor of cytokine production, but PGE<sub>2</sub> concentrations were not significantly different in mononuclear cell cultures from the vitamin E groups, compared with placebos. The lack of effect of vitamin E supplementation on PGE<sub>2</sub> production was unexpected, because we had found significant inhibition of PGE<sub>2</sub> production by vitamin E in a previous study (23). The difference may be

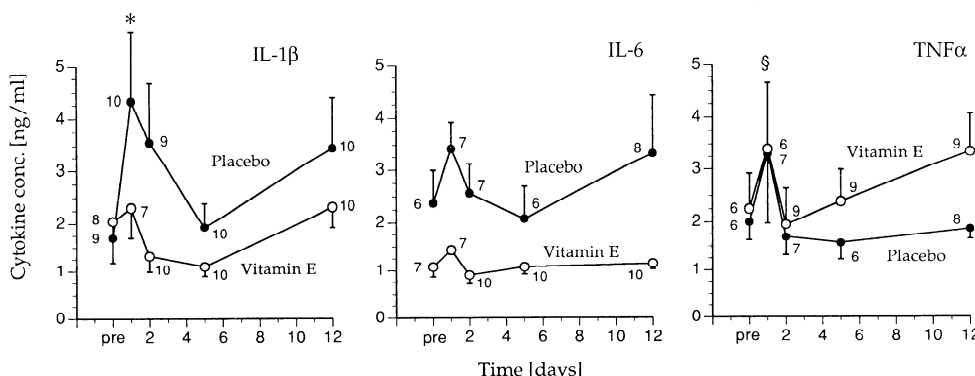


FIG. 1. Cytokine secretion by mononuclear cells isolated from morning blood samples. Cells were stimulated in vitro with 1 ng/ml lipopolysaccharide. Nos. next to each data point indicate sample size. IL, interleukin; TNF $\alpha$ , tumor necrosis factor- $\alpha$ . \*Significant increase ( $P < 0.05$ ) over preexercise (placebo group only); §significant increase ( $P < 0.05$ ) over preexercise (both groups combined).

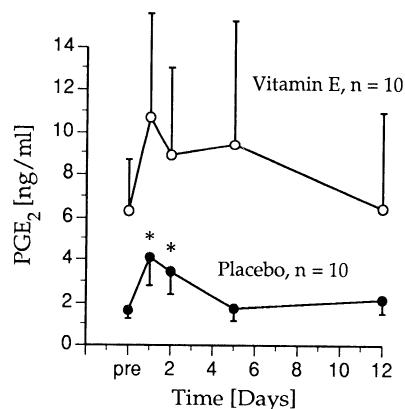


FIG. 2. Prostaglandin  $E_2$  ( $PGE_2$ ) secretion from mononuclear cells. \*Significant increase ( $P < 0.05$ ) over preexercise (placebo group only).

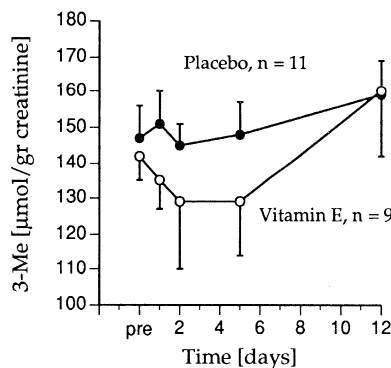


FIG. 3. Daily (24 h) urinary 3-methylhistidine (3-Me) excretion normalized to creatinine excretion.

related to the effectiveness of the supplementation: plasma levels of vitamin E increased 177% in the previous study and only 64% in the present study (6).

Dietary vitamin E also influences the lipoxygenase pathway of arachidonic acid metabolism leading to leukotrienes (16). Leukotrienes, in turn, stimulate mononuclear cell production of IL-1 $\beta$  (29). However, our evaluation of the role of leukotrienes was inconclusive, because the LTB $_4$  concentrations measured in the mononuclear cell cultures were at a level (1–10 pg/ml, data not shown) that might be attributable to cross-reaction with the prostaglandins in the cultures.

Oxygen radicals enhance endotoxin-induced IL-1 (19) and TNF- $\alpha$  (8) production. Furthermore, the concentrations of these reactants increase with exercise (10, 28). Thus the IL-1 $\beta$  responses observed in the present study are consistent with a mechanism involving oxygen radicals: exercise-induced oxygen radicals enhance endotoxin-induced IL-1 $\beta$  secretion after exercise in cells from placebo subjects, but not from the subjects taking the antioxidant vitamin E. However, the lack of effect on TNF- $\alpha$  secretion by vitamin E cannot be reconciled by this oxygen radical mechanism. The influence of oxygen radicals on IL-6 secretion has not been reported, to our knowledge.

**Plasma cytokines.** We have previously reported increased IL-1 activity after exercise using *in vivo* and *in vitro* bioassays (3, 5). In the present investigation, we employed more quantitative and specific immunoassays because it is now known that different cytokines share

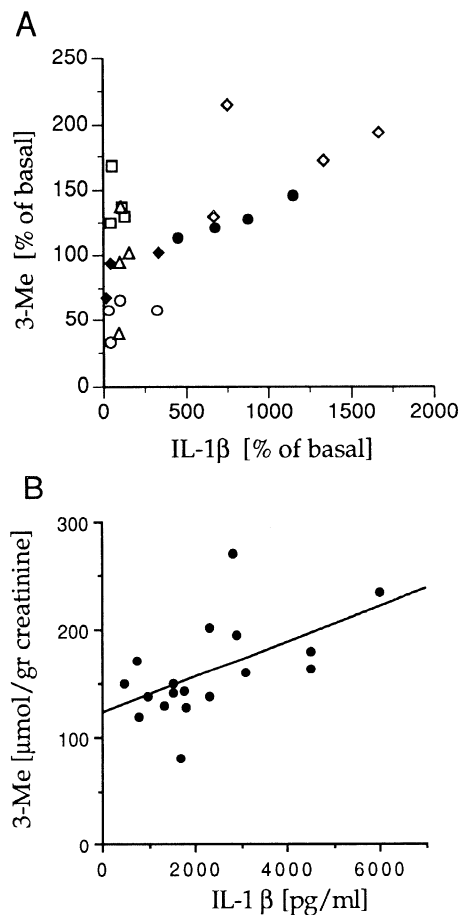


FIG. 4. Correlations between urinary 3-methylhistidine excretion and *in vitro* secretion of IL-1 $\beta$  from mononuclear cells. A: longitudinal data from 6 subjects exhibiting  $>35\%$  changes from preexercise baseline. Each subject is represented by a different symbol. Data at 1, 2, 5, and 12 days after exercise are expressed as a percentage of each subject's preexercise baseline value;  $\rho = 0.418$ ,  $P < 0.05$ . B: cross-sectional data at 12 days for all subjects;  $\rho = 0.479$ ,  $P < 0.05$ .

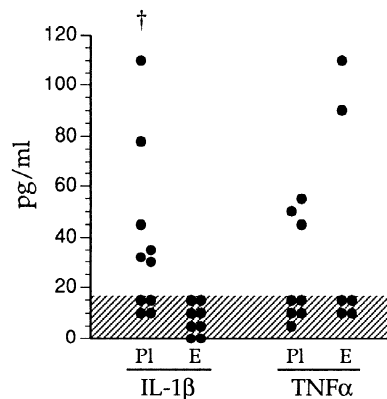


FIG. 5. Plasma IL-1 $\beta$  and TNF- $\alpha$  concentrations 6 h after eccentric exercise. All data points within crosshatched area represent plasma samples with undetectable concentrations. †Significant difference ( $P < 0.05$ ) between placebo and vitamin E groups.

many biological activities (11) and act synergistically in bioassays (18). The immunoactive plasma IL-1 $\beta$  observed in this investigation is consistent with previous observations of peak biological activity in plasma  $\sim 6$  h after exercise (3).

The quantitative immunoassays make it possible to compare the magnitude of the cytokine response after

TABLE 1. Incidence of immunoreactive plasma IL-1 $\beta$  and TNF $\alpha$ 

|                          | IL-1 $\beta$ |     | TNF $\alpha$ |     |
|--------------------------|--------------|-----|--------------|-----|
|                          | PI           | E   | PI           | E   |
| Preexercise              | 2/10         | 0/8 | 3/8          | 0/6 |
| Immediately postexercise | 3/10         | 0/8 | 1/8          | 1/6 |
| 3 h postexercise         | 3/10         | 0/8 | 1/8          | 1/6 |
| 6 h postexercise         | 6/10         | 0/8 | 3/8          | 2/6 |
| 24 h postexercise        | 0/10         | 0/8 | 1/8          | 0/6 |

PI, placebo; E, vitamin E group.

exercise to cytokine levels found during infection or an inflammatory response. At 6 h after exercise, the median IL-1 $\beta$  concentration in placebo subjects was 35 pg/ml, compared with 115 pg/ml observed in septic shock and 50 pg/ml induced in humans by experimental endotoxin infusion (7). The median TNF- $\alpha$  concentration after exercise was <10 pg/ml, whereas 110 and 890 pg/ml were observed in septic shock and endotoxin infusion, respectively (7).

The plasma cytokine data are consistent with the cytokine secretion observed in vitro in that plasma IL-1 $\beta$ , but not TNF- $\alpha$ , concentrations were significantly lower in the subjects taking vitamin E. However, the temporal patterns of plasma cytokine concentrations and cytokine secretion in vitro were different: plasma cytokine values were greatest at 6 h postexercise, but in vitro secretion was greatest at 24 h, when almost all plasma samples were negative for cytokine immunoreactivity. This discrepancy may be reconciled if several sources contribute to the circulating cytokine pool and if cytokine clearance increases along with cytokine production. Viti et al. (33) have suggested that increased cytokine activity after exercise might result from increased lymphatic drainage. Furthermore, Fong et al. (15) demonstrated that the TNF- $\alpha$  and IL-6 concentrations in the blood leaving the hepatic circulation were higher than systemic concentrations, consistent with the concept that fixed hepatic macrophages were secreting cytokines into the circulation. Michie et al. (25) have shown that during a continuous, 24-h infusion of TNF- $\alpha$  into humans, circulating TNF- $\alpha$  can only be detected for a few hours, suggesting that the cytokine upregulated its own clearance mechanisms.

**Protein metabolism.** A significant longitudinal correlation was observed between changes in IL-1 $\beta$  secretion and changes in 3-methylhistidine excretion over the 12-day period of observation, and significant cross-sectional correlations were found between 3-methylhistidine excretion and both IL-1 $\beta$  and PGE<sub>2</sub> secretion at 12 days. IL-1 $\beta$  is thought to be a "muscle proteolysis-inducing factor" active during infection and trauma. Although its actions have been inconsistent in vitro (2, 9, 17, 26), in vivo experiments indicate that IL-1 activates branched-chain  $\alpha$ -keto acid dehydrogenase (27) (a rate-limiting enzyme for amino acid oxidation in skeletal muscle) and increases muscle protein breakdown, as determined by leucine tracer studies (14). The results of the present investigation are consistent with the concept that mononuclear cell products, including PGE<sub>2</sub> and IL-1 $\beta$  are regulators of muscle proteolysis.

A hypothetical sequence of events after damaging exercise may begin with local disruption of tissue due to physical overload or oxidative stress. The resulting tissue fragments activate the complement system that in turn primes monocytes for activation by endotoxin (31) (as measured in this study) or by fragments of damaged tissue that may be encountered in vivo. The prolonged effect of eccentric exercise on urinary 3-methylhistidine excretion (13) may be related to the rate of mononuclear cell infiltration into muscle tissue. Round et al. (30), using downhill walking and other forms of eccentric exercise, found greater infiltration of mononuclear cells in biopsies taken 12–14 days after the exercise than biopsies taken at earlier times. Immunohistochemical analysis of IL-1 $\beta$  in muscle biopsies after eccentric exercise indicated that some subjects exhibited peak IL-1 $\beta$  content immediately after exercise, but in others IL-1 $\beta$  content was still increasing 5 days after exercise (4).

We have previously reported the changes in neutrophil numbers and function that occurred in the subjects described in the present investigation and how these related to changes in muscle membrane permeability (6). No significant associations were found between the parameters in that report and those reported here. Taken together, these two reports provide evidence that exercise initiates an acute phase response that may contribute to metabolic alterations after exercise. In addition, dietary vitamin E had a significant influence on IL-1 $\beta$  and IL-6 production, an action that may be exploited in future investigations for determining the fundamental role of these cytokines in inflammatory diseases as well as muscle damage and repair.

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Address for reprint requests: J. G. Cannon, Human Physiology Laboratory, USDA Human Nutrition Research Center on Aging, Tufts University, 711 Washington St., Boston, MA 02111.

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