Acute phase response in exercise: interaction of age and vitamin E on neutrophils and muscle enzyme release

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Cannon, Joseph G., Scott F. Orencole, Roger A. Fielding, Mohsen Meydani, Simin N. Meydani, Maria A. Fiatarone, Jeffrey B. Blumberg, and William J. Evans. Acute phase response in exercise: interaction of age and vitamin E on neutrophils and muscle enzyme release. Am. J. Physiol. 259 (Regulatory Integrative Comp. Physiol. 28): R1214–R1219, 1990.—Several host defense responses and metabolic reactions that occur during infection have been observed after exercise. We hypothesized that these reactions, known as the “acute phase response,” contribute to the breakdown and clearance of damaged tissue after exercise. This hypothesis was tested with 21 male volunteers representing two ranges of age (22–29 and 55–74 yr), who ran downhill on an inclined treadmill to accentuate damaging eccentric muscular contractions. The subject groups were further divided in a double-blind placebo-controlled protocol, which examined the influence of 48 days of dietary vitamin E supplementation before the exercise. All subjects were monitored for 12 days after exercise for changes in circulating leukocytes, superoxide release from neutrophils, lipid peroxidation, and efflux of the intramuscular enzyme creatine kinase (CK) into the circulation. Among those receiving placebo, the <30-yr-old subjects responded to exercise with a significantly greater neutrophilia and higher plasma CK concentrations than the >55-yr-old subjects. Dietary supplementation with vitamin E tended to eliminate the differences between the two age groups, primarily by increasing the responses of the >55-yr-old subjects. At the time of peak concentrations in the plasma, CK correlated significantly with superoxide release from neutrophils. The association of enzyme efflux with neutrophil mobilization and function supports the concept that neutrophils are involved in the delayed increase in muscle membrane permeability after damaging exercise.

superoxide; creatine kinase; lipid peroxides; muscle damage

INJURY AND INFECTION initiate a stereotyped series of host defense reactions known collectively as the acute phase response. Neutrophils are mobilized and activated within the first few hours of the response. These cells phagocytize pathogenic organisms and destroy them with oxygen free radicals and nonspecific proteases. However, inappropriate release of these agents can damage host tissues and are thought to be the basis of noninfectious inflammatory diseases (22).

Heavy exercise induces several reactions suggestive of an acute phase response, including leukocytosis and increases in host defense mediators such as interleukin 1 (6). A particularly damaging form of exercise is eccentric exercise, in which a muscle is forced to lengthen as it develops tension (2). After eccentric exercise, delayed muscle soreness and infiltration of leukocytes have been reported (1, 18). We hypothesized that damaging exercise initiates an acute phase response that contributes to the breakdown and clearance of overloaded tissue. Evidence that lipid peroxidation products increase after exercise (8, 10) prompted us to explore the role of neutrophils and neutrophil-generated oxygen radicals in myocellular permeability after damaging eccentric exercise.

An eccentrically biased exercise protocol was performed by groups of subjects expected to express differing susceptibilities to exercise-induced or free radical induced damage. Endogenous levels of protective antioxidant enzymes reportedly decline with age (11); therefore subjects <30 yr of age were compared to subjects >55 yr of age. Some members of each age group were given dietary supplements containing the antioxidant vitamin E with the intention of reducing oxygen radical-induced metabolic changes. The subjects were monitored for 12 days after exercise for changes in circulating leukocytes, superoxide release by neutrophils, efflux of the intramuscular enzyme creatine kinase (CK) into the circulation, and lipid peroxidation. The results of the investigation indicate relationships do exist between neutrophils and muscle metabolism after exercise; however, the influence of age and dietary antioxidant supplementation was contrary to expectations.

METHODS

Human subjects protocol. Sedentary men representing two ranges of age (22–29 yr, n = 9; and 55–74 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 maximum oxygen consumption (VO_{2max}) test on a cycle ergometer. Using a random allocation system, four <30-yr-old and six >55-yr-old subjects supplemented their diet with dl-α-tocopherol (vitamin E) taken as capsules (400 IU in soybean oil twice daily, Hoffmann-La Roche,
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 α-tocopherol + γ-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 the supplements were 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 earlier. 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 anti-inflammatory or analgesic drugs throughout the course of the study. Needle biopsies were taken from the vastus lateralis muscles of the <30-yr-old 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.

Plasma α-tocopherol. Plasma was stored under nitrogen at -70°C for tocopherol analysis. The samples were analyzed by high-performance liquid chromatography (17) using a C18 reverse-phase column in 100% methanol. Tocotrienol was used as an internal standard for quantification (a gift from Hoffmann-La Roche).

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

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

Neutrophil isolation. Plasma was separated from heparinized blood by centrifugation. The packed cells were resuspended in culture medium (RPMI) and subjected to density-gradient centrifugation using ficoll-hypaque (Sigma, St. Louis, MO). The mononuclear cell and ficoll-hypaque layers were aspirated and the neutrophil-red cell pellet was recovered. The neutrophils were isolated by dextran sedimentation then washed and resuspended in Hanks’ balanced salt solution and incubated in 1.5 ml polypropylene microcentrifuge tubes at a final concentration of 2.5 \( \times \) 10^6 cells/ml.

Superoxide release from neutrophils. Superoxide was measured by the reduction of ferricytochrome c to ferrocytochrome c (3). The neutrophils were incubated for 20 min in a 37°C shaking water bath with 1.2 mg/ml cytochrome c (type VI, Sigma) under the following four experimental conditions: 1) with no other additives; 2) with 100 μg/ml superoxide dismutase (SOD, Sigma); 3) with 5 μg/ml phorbol 12-myristate 13-acetate (PMA, Sigma); or 4) SOD and PMA. After incubation, the cells were pelleted by centrifugation and the supernatants transferred to new microcentrifuge tubes and frozen until spectrophotometric analysis. Reduction of cytochrome c in each condition was determined by measuring the difference in absorbance at 535 and 555 nm. Basal superoxide concentration was calculated as the difference in cytochrome c reduction in conditions 1 and 2, multiplied by the molar extinction coefficient of 29.5 (as specified by Sigma) to yield a value in micromoles per milliliter. Stimulated superoxide production was calculated in a similar fashion using conditions 3 and 4.

Plasma lipid peroxides. Malondialdehyde, other aldehydes, and end products of lipid peroxidation were detected after reaction with thiobarbituric acid, which forms a red chromophore (31). The samples were then extracted with n-butanol and measured fluorometrically (515 nm excitation, 553 nm emission). 1,1,3,3-Tetramethoxypropane was used as a standard.

Statistical analyses. Values reported are means ± SE. Significant differences between groups were determined by one- and two-factor analysis of variance followed by Fisher’s least significant difference test. Plasma CK data were normalized by log transformation before analysis of variance. Associations between variables were investigated using the Spearman rank correlation coefficient (ρ).

RESULTS

Group characteristics. The aerobic capacities for the placebo subjects were not different from the vitamin E-supplemented subjects within each age group (Table 1). However, the <30-yr-old group had significantly higher aerobic capacities than the >55-yr-old group.

After 48 days of dietary supplementation, the mean plasma vitamin E concentrations for the groups taking vitamin E were significantly higher than the placebo groups (Table 1). In addition, the >55-yr-old subjects had significantly higher plasma vitamin E levels (P < 0.05) compared with the <30-yr-old subjects receiving the same supplement. The placebo groups exhibited no change in plasma tocopherol compared with presupplementation levels. No significant changes in plasma vitamin E concentration were observed during the 72-h period after exercise when the supplements were discontinued (data not shown).

Creatine kinase. Plasma CK data were normalized for intersubject variations in muscle mass by dividing the plasma CK concentration by the mass of creatinine excreted in the urine over 24 h (15). The creatinine excretion values used for this normalization were the
Table 1. Subject characteristics

<table>
<thead>
<tr>
<th></th>
<th>Placebo (n = 5)</th>
<th>Vitamin E (n = 4)</th>
<th>Placebo (n = 6)</th>
<th>Vitamin E (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( V_{\text{O}_2,\text{max}} ), ml·kg(^{-1})·min(^{-1})</td>
<td>45±1</td>
<td>42±2</td>
<td>31±2†</td>
<td>29±2†</td>
</tr>
<tr>
<td>Plasma ( \alpha )-tocopherol, ( \mu )g/ml</td>
<td>6.16±0.50</td>
<td>9.05±2.88</td>
<td>10.59±1.48†</td>
<td>10.14±2.67</td>
</tr>
<tr>
<td>Presupplement</td>
<td>6.32±0.95</td>
<td>14.16±2.40†</td>
<td>10.33±1.02†</td>
<td>17.36±1.91†</td>
</tr>
<tr>
<td>Postsupplement</td>
<td></td>
<td></td>
<td></td>
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</tbody>
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Values are means ± SE; \( n \), no. of subjects. \( V_{\text{O}_2,\text{max}} \), maximum \( \text{O}_2 \) consumption. * Significantly higher than placebo subjects in same age group, \( P < 0.05 \). † Significantly lower than <30-yr-old subjects, \( P < 0.05 \). ‡ Significantly higher than <30-yr-old subjects in same dietary group, \( P < 0.05 \).

The mean plasma CK concentrations in all four groups were significantly increased the morning after exercise (\( P < 0.01 \), Fig. 1). The <30-yr-old placebo-treated subjects increased to a maximum of 295 ± 108 IU·l\(^{-1}\)·g creatinine\(^{-1}\) at this time. The peak increase was similar in the <30-yr-old subjects taking vitamin E (322 ± 88 IU·l\(^{-1}\)·g creatinine\(^{-1}\)), but CK levels tended to return to baseline levels more rapidly. The plasma CK concentrations in the >55-yr-old placebo-supplemented subjects only reached a maximum of 117 ± 33 IU·l\(^{-1}\)·g creatinine\(^{-1}\), but those receiving vitamin E exhibited plasma CK levels similar to the <30-yr-old subjects (294 ± 94 IU·l\(^{-1}\)·g creatinine\(^{-1}\)).

Plasma CK concentrations in the >55-yr-old subjects taking vitamin E were significantly higher than in the >55-yr-old placebo subjects before exercise and 2 days after exercise. The differences between the plasma CK levels in the <30-yr-old and >55-yr-old placebo groups were statistically significant (\( P < 0.05 \)) before, 2, and 5 days after exercise. Vitamin E supplementation tended to reduce CK levels in <30-yr-old subjects and increase them in >55-yr-old subjects on days 2 and 5.

Circulating neutrophils. Exercise-induced changes in neutrophils exhibited age- and vitamin E-related patterns similar to those observed for plasma CK. Six hours after downhill running, the placebo- and vitamin E-supplemented <30-yr-old subjects exhibited peak increases in circulating neutrophils (5,200 ± 860 and 5,600 ± 1,250 cells/mm\(^3\), respectively, \( P < 0.01 \), Fig. 2). The >55-yr-old group receiving placebo exhibited a much smaller increase in neutrophil counts, peaking 3 h after exercise at 3,280 ± 220 cells/mm\(^3\) (\( P < 0.01 \)). The neutrophil counts in the vitamin E-supplemented >55-yr-old group were similar to the <30-yr-old group, reaching 5,260 ± 360 cells/mm\(^3\) at 6 h (\( P < 0.01 \)).

In the days after eccentric exercise, vitamin E supplementation had divergent effects on neutrophil counts, depending on the age of the subjects; the >55-yr-old vitamin E-supplemented subjects had higher neutrophil counts compared with placebo, and <30-yr-old vitamin E-supplemented subjects had lower counts than placebo (Table 2). No differences in the number of immature band cells were observed at any of the time points or between any of the groups.

Superoxide release from neutrophils. Superoxide release was measured immediately after and 3 and 6 h after exercise in the first five subjects entered in the study (1 in each group, except 2 in >55-yr-old placebo group). No changes were observed during this period of time (data not shown); therefore, for the remaining subjects, the first postexercise superoxide determination was made 24 h after exercise. Basal superoxide secretion by neutrophils (i.e., no exogenous stimulus) tended to increase over baseline at 24 h in all groups (Table 3), but these increases were not significant. In the >55-yr-old groups, superoxide release from neutrophils taken at later time points exhibited higher levels, but only the placebo group attained levels significantly higher than preexercise. The capacity of the cells to release superoxide in response to a supramaximal stimulus (5 \( \mu \)g/ml PMA) was similar in all groups before exercise and unchanged after exercise up to 5 days (6.87 ± 0.85 \( \mu \)mol/ml). Twelve days after

![FIG. 1. Plasma creatine kinase (CK) concentrations in morning blood samples over 12 days of observation. A: responses of <30-yr-old subjects (\( n = 5 \) placebo, \( n = 4 \) vitamin E). B: responses of >55-yr-old subjects (\( n = 5 \) placebo, \( n = 6 \) vitamin E). One >55-yr-old placebo subject is omitted from analysis because of missing data at day 1. *Significant increases from preexercise (\( P < 0.01 \)); †significant differences between >55-yr-old placebo and vitamin E groups (\( P < 0.05 \)); ‡significant differences between <30-yr-old and >55-yr-old placebo groups (\( P < 0.05 \)).](image-url)
exercise, the neutrophils from both groups of >55-yr-old subjects exhibited an increased response to PMA (9.74 ± 1.45 μmol/ml, P < 0.05).

The observation that vitamin E supplementation of the >55-yr-old subjects promoted increases in both circulating neutrophils and plasma CK prompted us to examine the statistical relationship between neutrophil function and myocellular enzyme efflux. A significant correlation (ρ = 0.751, P < 0.001) was found between basal superoxide release from neutrophils and plasma CK at the time of peak CK concentrations (1 day after exercise, Fig. 3). CK release did not correlate with the number of circulating neutrophils.

**Lipid peroxides.** Plasma lipid peroxide concentrations increased in 18 of the subjects within 24 h of exercise with a mean increase of 30 ± 8%. However, the time this increase occurred varied considerably between individuals, resulting in no statistically significant increase at any particular time point (Table 4).
DISCUSSION

The most striking findings in this investigation were the influence of age and vitamin E supplementation on the increases in circulating neutrophils and plasma CK concentrations after exercise. These responses, which have often been observed in young subjects (25, 30), were diminished in the >55-yr-old subjects receiving placebo and seemed to be "restored" by dietary vitamin E supplementation. Vitamin E maintains membrane fluidity and stability and protects the lipid bilayer against free radical injury and lipid peroxidation (23, 29). Therefore, the increased CK release in >55-yr-old subjects supplemented with vitamin E appears to contradict the hypothesis that exercise-induced changes in muscle membrane permeability are the result of damage by oxygen radicals. Indeed, the results conform more closely to two alternative hypotheses. The first hypothesis states that muscle enzyme release occurs after reversible formation of membrane pores (32), possibly as a mechanism for controlling cell volume (9). In this situation, vitamin E-enhanced membrane fluidity might be expected to promote enzyme release. The second hypothesis is based on the observation that inhibitors of lipoxygenase enzymes reduce efflux of CK from damaged muscle in vitro (16), indicating that products of the lipoxygenase pathway may mediate changes in muscle membrane permeability. Activated neutrophils are a prime source of lipoxygenase products and α-tocopherol has been shown to have a biphasic effect on neutrophil lipoxygenase activity: concentrations up to ~25 μg/ml enhance and higher concentrations inhibit the enzyme activity (12). Based on these data, the neutrophils from our vitamin E-supplemented subjects would be expected to have higher lipoxygenase activity, and this may have contributed to increased muscle membrane permeability.

CK concentrations in the plasma correlated with superoxide release from neutrophils at the time of peak CK levels (Fig. 3), but significant increases in plasma lipid peroxides were not observed. It is possible that superoxide is not responsible for the alteration in muscle membrane permeability, but instead indicates neutrophil activation and release of lipoxygenase products or catabolic enzymes. However, a mechanism involving membrane damage by oxygen radicals cannot be completely discounted. The paradoxical effect of vitamin E on the >55-yr-old subjects might be reconciled if one considers that neutrophils are damaged by their own oxidative products and are protected by vitamin E supplementation (4). The diminished neutrophilia after exercise in the >55-yr-old placebo group may reflect greater autooxidation of neutrophils that have become more vulnerable to oxidative damage with age (13). By protecting neutrophils, producers of free radicals, vitamin E may indirectly promote a net increase in muscle membrane damage, even though it has a direct protective effect on the muscle membrane itself.

The magnitude of superoxide release per neutrophil is clearly not the critical determinant of enzyme efflux because some of the highest superoxide levels were observed in >55-yr-old subjects 5 and 12 days after exercise when CK levels had returned to baseline. Instead, a coordinated neutrophil response involving mobilization into the circulation, followed by localization and activation in affected muscle tissue may be required for muscle enzyme efflux.

The time course of the observed changes in circulating neutrophils and subsequent changes in muscle membrane permeability are consistent with the life cycle of the neutrophil. About one-half of the mature neutrophils released from the bone marrow are sequestered (marginated) along blood vessel walls. Many factors, including epinephrine, increased blood flow, and inflammatory mediators will promote release of these neutrophils into the circulation. Neutrophil half life in the circulation is ~10 h. The cells then egress into tissue, drawn to specific sites by products of inflammation or infection (chemotaxis), then release free radicals and degradative enzymes such as elastase and lysozyme. Neutrophils are thought to live only 1 or 2 days after migrating into tissue (5).

Although not measured in this investigation, vitamin E supplementation may have promoted localization of neutrophils in overloaded or ischemic muscle tissue. Antioxidants have been shown to increase human neutrophil chemotaxis in vitro (4). In vivo, the suppressed chemotaxis observed in vitamin E-deficient rats was restored by parenteral administration of vitamin E (14). Therefore, after damaging exercise, vitamin E may promote neutrophil accumulation at specific sites of tissue damage. A highly focused action by activated neutrophils may explain why no significant changes in lipid peroxidation were detected systemically (Table 4). Clearance rates of lipid peroxides may be sufficiently rapid that recirculated blood sampled from the antecubital vein is a poor measure of lipid peroxide generation in leg muscles.

Previous studies have linked postexercise free radical accumulation, lipid peroxidation, and membrane damage to reduced mitochondrial respiratory control (8, 26). The results of the present investigation indicate that neutrophils may be an additional source of free radicals after exercise. It has been reported that neutrophils also release elastase (20) and lysozyme (24) after exercise, which could also contribute to tissue catabolism. The action of neutrophils after exercise may be similar to ischemia reperfusion injury of skeletal and cardiac muscle. Ischemia and subsequent reperfusion result in reduced function, increased histological evidence of cellular damage, and increased release of CK and other cytoplasmic enzymes (19). During reperfusion, an increase in tissue neutrophil content has been observed (28). In isolated, perfused organ systems, reperfusion after ischemia with neutrophil-containing buffer caused more histological and functional damage than buffer alone, indicating a causal association (21).

The finding that CK release was more pronounced in young people, who are usually considered to be more resilient to physical stress than older subjects, calls into question the assumption that the efflux of myocellular enzymes represents undesirable "damage" to muscle membranes. An alternate hypothesis is that CK is one manifestation of increased muscle protein turnover, which is necessary to clear partially damaged proteins. Salminen and Vihko (27) put forward a similar hypoth-
nesis after finding that the acid proteolytic capacity of young mice increased to a greater extent than in older mice after prolonged exercise. "Aging," therefore, may be the buildup of dysfunctional proteins through a reduction of necessary clearance mechanisms. This concept is similar to the pathogenesis of amyloidosis, a buildup of insoluble fibrillar protein that can manifest itself in a number of age-related diseases, including endocrine tumors and neuropathies (7).

In summary, the data from the present investigation indicate that mobilization and activation of neutrophils may contribute to increased myocellular enzyme efflux after eccentric exercise. Furthermore, this investigation shows that age-related differences in response can be modulated by dietary supplementation.

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We refer the reader to the original references for further details.

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REFERENCES


