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Rapid Letter

Physical Exercise Induces Activation of NF-κB in Human Peripheral Blood Lymphocytes

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ABSTRACT

Current understanding of nuclear factor-κB (NF-κB) activation is derived mostly from *in vitro* studies, and *in vivo* human data are limited. This study provides first evidence showing that physical exercise (80% maximal O_2 consumption, 1 h) may trigger NF-κB activation, as determined by electrophoretic mobility shift assay, in peripheral blood lymphocytes of physically fit young men. Supershift assay showed that the NF-κB protein complex contained the transcriptionally active p65 protein. Plasma levels of NF-κB-directed gene products such as tumor necrosis factor-α and interleukin-2 receptor confirmed that physical exercise caused NF-κB transactivation. Exercise-induced NF-κB activation in lymphocytes was associated with elevated levels of lipid peroxidation by-products in the plasma. Antioxid. Redox Signal. 3, 1131–1137.

INTRODUCTION

THE NUCLEAR FACTOR-κB (NF-κB) p50/p65 heterodimer is the classical member of the Rel family of transcription factors that regulate diverse cellular functions such as immune response, cell growth, survival, and development (2, 3, 7, 33). In lymphocytes, NF-κB activation represents a normal component of cell response to a wide variety of stimuli (2, 6, 16, 28, 30). NF-κB regulates cell division, apoptosis, and differentiation that accompany lymphocyte activation (6). NF-κB activity is induced by a wide variety of factors, including cytokines, phosphatase inhibitors, endotoxin and certain protein phosphorylation agonists (2, 3). Oxidants have been shown to induce NF-κB activation in several types, and it has been postulated that oxidants may serve as intracellular messengers inducing NF-κB activity (14, 21, 30, 33).

Strenuous or prolonged exercise is known to induce oxidative stress (29, 31, 32). In humans, physical exercise has been observed to induce oxidative DNA damage in lymphocytes (11). It has been suggested that neutrophil-derived oxidants cause such damage (22). Most of our current knowledge regarding the various factors that induce NF-κB activity is derived from *in vitro* studies (2, 3, 7). Information from human *in vivo* studies is limited. In this study, we sought to investigate whether strenuous prolonged exercise influences lymphocyte NF-κB activity in physically fit men.

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MATERIALS AND METHODS

Subjects

Twelve healthy male endurance and mixed-aerobic athletes (mean age, 24.2 ± 6.5 years, body mass index, 22.2 ± 1.2) were studied after giving informed consent. These athletes included five international class cross-country skiers, four national class middle- and long-distance runners, one semicompetitive distance runner, one rower, and one national class basketball player. The study was performed with the approval of the Human Ethics Committee of the University of Tartu (Tartu, Estonia).

Protocol for exercise

Subjects underwent a maximal exercise test to determine maximal O_2 consumption (VO_{2max}). They performed an incremental treadmill (LE 3000) exercise test until volitional exhaustion. VO_{2max} was measured using "breath by breath" gas monitoring (Oxycon Record, Erich Jaeger, Germany). One week later, all subjects exercised for 60 min at 80% of their VO_{2max} after a 5-min warm-up. Subjects were asked to refrain from intensive exercise for at least 3 days preceding the test. On the day of exercise testing, the subjects ate a light carbohydrate-rich breakfast. The exercise tests were carried out 2–4 h after breakfast.

Blood sampling

Blood was drawn from an antecubital vein before and immediately after sustained exercise at 80% VO_{2max}.

Routine blood analyses

Complete blood cell counts, including total leukocyte, neutrophil, lymphocyte, and monocyte counts, hemoglobin, and hematocrit were measured from EDTA-treated blood using an automated hematology analyzer (Sysmex-SE9000, Japan).

Determination of lymphocyte subpopulations

Flow cytometry was performed to determine the lymphocyte subpopulations (12). Whole blood leukocytes were labeled with monoclonal antibodies against surface determinants to identify the $CD3^+$ (T-cell), $CD19^+$ (B-cell), and $CD16^+CD56^+/CD3^-$ (natural killer cell) lymphocyte subpopulations. All antibodies were purchased from Becton–Dickinson (San Jose, CA, U.S.A.). Data were acquired and analyzed on a FACSort flow cytometer by two-color flow cytometric analysis using SimulSET software (Becton–Dickinson, Palo Alto, CA, U.S.A.).

Tumor necrosis factor-α (TNFα) and interleukin 2 receptor (IL-2R) assays

IMMULITE TNFα and IMMULITE IL-2R solid-phase, two-site chemiluminescent enzyme immunometric assays (DPC, U.K.) for use with the IMMULITE Automated Analyzer were used for the quantitative measurement of plasma TNFα and soluble plasma IL-2R (1, 4, 5).

Lymphocyte isolation and assay of NF-κB activation

Lymphocytes were isolated from fresh whole blood using Ficoll-Paque PLUS (Pharmacia, 17-1440-02) and low-speed centrifugation as described by others (8). The isolated suspension of lymphocytes was subjected to whole-cell protein extraction for the determination of NF-κB as described before (27). In brief, total cell extracts were prepared using a high-salt detergent buffer. Lymphocytes were resuspended and lysed in 0.050 ml of buffer containing 20 m*M* HEPES, pH 7.5, 400 m*M* NaCl, 1 m*M* $MgCl_2$, 0.5 EDTA, 20% glycerol, 1% Nonidet P-40, 0.5 m*M* dithiothreitol, 0.010 mg/ml leupeptin, 1 m*M* phenylmethylsulfonyl fluoride, and 0.001% aprotinin. The extracts were centrifuged for 15 min at 14,000 rpm in a microcentrifuge at 4°C, and clear supernatant was stored at −70°C. Protein concentration in the supernatant was determined using the Bradford protein assay reagent (Bio-Rad, Hercules, CA, U.S.A.). The samples were subjected to electrophoretic mobility shift assay for the determination of NF-κB activity (27).

Binding reaction mixtures contained 0.02 mg of extracted protein, 0.002 mg of ds+sDNA as nonspecific competitor, 0.002 mg of bovine serum albumin, 20 m*M* HEPES, pH 7.5, 50 m*M* NaCl, 0.5 m*M* $MgCl_2$, 2% glycerol, and 0.25 ng of ^{32}P-end-labeled NF-κB specific doublestranded oligonucleotide probe (Santa Cruz Biotechnology Inc., Santa Cruz, CA, U.S.A.). To determine the specificity of the NF-κB band, a competition assay (13) was performed using a 20-fold excess of the unlabeled probe. To assess the subunit composition of DNA binding protein, specific antibodies were used for supershift assay (13). Cellular extracts were incubated with antibodies against p65 (Santa Cruz Biotechnology Inc.) subunits of NF-κB for 15 min at room temperature before the binding reaction mixture was added. After the binding reaction (30 min at room temperature), the samples were run on a native 4.8% polyacrylamide gel in 0.5× TBE buffer (12.5 m*M* Tris-borate containing 0.25 m*M* Na_2EDTA, pH 8.0). NF-κB bands were detected by autoradiography and quantified by densitometry (13).

Lipid peroxidation markers

Serum conjugated diene (CD) levels were measured according to methods previously described (25) with minor modifications (35). In brief, 0.25% butylated hydroxytoluene (BHT)-treated serum samples (0.15 ml) and 0.15 ml of 0.9% NaCl were incubated at 37°C for 25 min, and lipids were extracted by heptane/isopropanol (1:1). Samples were acidified by 5 mol/L hydrochloric acid and extracted by cold heptane. After centrifugation for 5 min at 3,000 rpm, the absorbance of the heptane fraction was measured at the absorbance maximum between 220 and 250 nm using isotonic saline as blanks.

Conversion of conjugated dienes results in stable secondary products (aldehydes, alkenals) of lipid peroxidation. The assay for thiobarbituric acid (TBA) reactive products (TBARS), in which TBA reacts with malondialdehyde, is a sensitive but relatively nonspecific method for detection of later stage lipid peroxidation. TBARS levels were detected in serum samples (23) with minor modifications to increase specificity (35). In brief, samples were treated with BHT twice, immediately after collection and before addition of the test reagents, to suppress oxidative changes during handling and analysis. Hemolyzed samples were excluded from analysis. Samples (0.25 ml) were incubated with 0.475 m*M* Fe^{2+} at 37°C for 30 min. After incubation, BHT (0.25%) was added to the samples. This mixture was treated with acetate buffer, pH 3.5, and heated with TBA solution (1%, 80°C, 70 min). The samples were then cooled and acidified (5 mol/L hydrochloric acid). After extraction with cold butanol, samples were centrifuged. The absorbance of the butanol fraction was measured at 534 nm. A standard plot for malondialdehyde (end-product of lipid peroxidation) was prepared using 1,1,3,3-tetraethoxypropane.

Statistical analysis

All results are presented as means ± SD. TNFα and TBARS levels were log-transformed before statistical analyses to correct for skewing, although raw values are presented in the results. ANOVA for repeated measures was used for analyzing the change of variables with exercise. Results were adjusted as necessary for hemoconcentration by using changes in hematocrit with exercise as a covariate. In all analyses, a value of $p < 0.05$ was considered statistically significant.

RESULTS

The mean VO_{2max} (68.6 ± 7.6 ml/min/kg) indicated that the men were highly fit. Both the hemoglobin concentration and hematocrit rose slightly with exercise (Table 1, p = 0.035–0.039).

Sixty minutes of strenuous cycling exercise induced moderate leukocytosis in the peripheral blood stream (Table 1). Neutrophilia was most prominent (p = 0.003). The total peripheral lymphocyte count tended to increase (p = 0.058), but not after controlling for plasma volume contraction as estimated by the change in hematocrit. On the other hand, the natural killer cell lymphocyte subpopulation showed a marked increase with exercise (p = 0.007). Monocyte levels remained unchanged.

Physical exercise caused activation of NF-κB in eight of 12 individuals. No appreciable change was detected in three men, and in one case (subject 4, Fig. 1) preexercise baseline activity was higher than postexercise activity (Fig. 1). Supershift assay using anti-p65 antibody (lanes 1 and 2 from left; Fig. 1A) showed

TABLE 1. PERIPHERAL WHITE BLOOD CELL COUNTS, PLASMA LEVELS OF TNF-α, SOLUBLE IL2-R, AND LIPID PEROXIDATION IN 12 YOUNG MEN JUST BEFORE AND JUST AFTER 60 MIN OF EXERCISE AT 80% VO_{2MAX}

	Preexercise	*Postexercise*	p
Hemoglobin (g/L)	153 ± 13.04	160 ± 9.0	0.035
Hematocrit (%)	0.435 ± 0.04	0.45 ± 0.02	0.039
Leukocytes			
Total white blood cells (cells × 10^9/L)	5.56 ± 1.11	8.10 ± 2.15	0.001
Lymphocytes (cells × 10^9/L)	1.49 ± 0.48	1.76 ± 0.38	0.058
T-cells (cells × 10^9/L)	1.05 ± 0.36	1.14 ± 0.29	0.404
B-cells (cells × 10^9/L)	0.174 ± 0.05	0.192 ± 0.02	0.440
Natural killer cells (cells × 10^9/L)	0.26 ± 0.16	0.44 ± 0.23	0.007
Neutrophils (cells × 10^9/L)	3.23 ± 0.81	5.60 ± 2.11	0.003
Monocytes (cells × 10^9/L)	0.57 ± 0.16	0.55 ± 0.15	0.670
Cytokine and receptor			
TNFα (pg/ml)	21.8 ± 6.3	25.7 ± 5.2	0.003
Soluble IL-2R (U/ml)	543 ± 174	577 ± 195	0.005
Lipid peroxidation			
TBA reactivity (μM malondialdehyde equivalents)	1.63 ± 0.38	1.93 ± 0.28	0.009
Conjugated dienes (μM)	50.94 ± 10.14	56.39 ± 10.86	0.047

Data are means ± SD. See Materials and Methods for statistical treatment.

that the NF-κB protein complex contained the transcriptionally active p65 protein. When the one case of exceptional outlying response (subject 4, Fig. 1B) was excluded from analyses, the mean NF-κB activity increased by >50% (p = 0.002) as detected from densitometry data.

Strenuous exercise increased plasma TNFα (p = 0.003) and soluble plasma IL-2R (p = 0.005) levels (Table 1). These increases were significant even after controlling for changes in hematocrit. Plasma conjugated diene (p = 0.047) and especially TBARS (p = 0.009) concentrations increased with exercise (Table 1).

DISCUSSION

In humans, physical exercise markedly influences immune function (34, 39). In physically fit men, strenuous exercise for 1 h increased total white blood cell count in the peripheral blood. In 1893, Schultz first described that exercise may cause leukocytosis (26). Several studies have repeated this observation (39). Physical exercise is also known to influence the composition of the lymphocyte subset in peripheral blood (9, 10, 18, 19). The nature of this effect is thought to be dependent

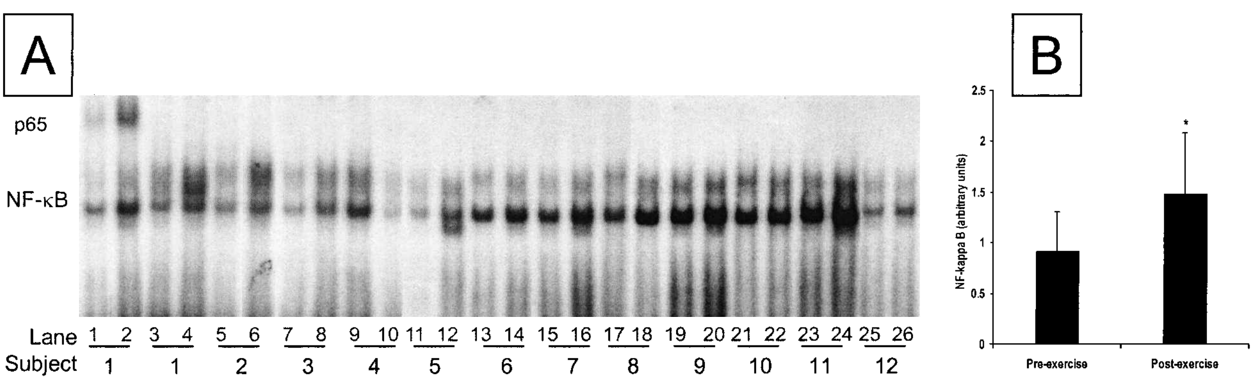

FIG. 1. DNA-binding of NF-κB harvested from peripheral blood lymphocytes of 12 young men before and after 60 min of exercise at 80% VO_{2max}. Lanes 1 and 2, supershift evidence; lanes 3 and 4, preexercise and postexercise from subject 1, respectively; lanes 5 and 6, subject 2; etc. The first two lanes show supershift data for the presence of p65. (**B**) Densitometry data. Units are arbitrary, with preexercise levels set at 1.0. p for the difference between before and after exercise was 0.061. After omission of an outlying response (subject 4), the difference was highly significant (p = 0.002).

on the exercise type and intensity. Exercise-induced changes in the lymphocyte subset pattern are not related to changes in blood volume, suggesting that a bout of exercise directly influences lymphocyte function and fate (15). In response to the exercise test used in the current study, no significant changes were observed in total lymphocyte or monocyte count, T-cell count, or B-cell count. Consistent with previous reports (20), the count of natural killer lymphocytes in the peripheral blood markedly increased in response to exercise. An increase in plasma epinephrine during exercise is thought to be one factor responsible for the exercise-induced increase in peripheral blood natural killer cell count (24). Neutrophilia is known to occur in response to endurance exercise (36). We observed that the 1-h bout of exercise markedly enhanced the peripheral blood neutrophil count. Exercise-induced changes in peripheral blood cell counts observed in this study represented typical changes that are expected in response to endurance exercise.

Results of this study provide first evidence that strenuous exercise in well trained young men may cause activation of NF-κB. The NF-κB activation process is known to be transient (2, 3, 7, 30). Activated NF-κB signals for the expression of the inhibitor protein IκB. IκB, thus expressed, terminates NF-κB activation and resets the NF-κB activation switch in the cytosol (2, 3, 7, 30). This transient nature of NF-κB activation may explain the lack of exercise-induced NF-κB activity in three of 12 men. Perhaps the NF-κB response kinetics in these men were such that they were not detectable at the time point the samples were collected. Basal NF-κB activity is usually low, and the activity of this transcription factor is known to be induced by a wide variety of stimuli (2, 3, 7, 30). Consistently, we have observed that NF-κB activity in preexercise baseline samples was low in 11 of 12 subjects. In the one exceptional case, a factor unknown to us contributed to high baseline NF-κB activity. Consistent with the electrophoretic mobility shift assay, physical exercise also enhanced the expression of NF-κB-directed gene products TNFα and IL2-R (2, 3, 7, 30). This effect was observed in each and every subject studied (individual data not shown). This line of evidence is consistent with our observation that physical exercise-induced activation of NF-κB proteins includes the transcriptionally active p65 protein. It is therefore evident that exercise-induced cytosolic activation of NF-κB is associated with a transactivation response. The results presented, however, do not allow delineation of the mechanism of exercise-induced NF-κB activation. It is possible, however, that the effect is triggered by a combination of multiple factors. In this study it was observed that exercise caused oxidative stress in the blood as evident from lipid peroxidation data. At the same time, exercise elevated plasma levels of TNFα. Both reactive oxygen species and TNFα are thought to be potent activators of NF-κB (14, 21, 30, 33). It has been also demonstrated that TNFα signaling may involve reactive oxygen species as intracellular messengers (17, 37, 38). It is thus of interest to examine whether exercise-induced NF-κB activation is inhibited by dietary antioxidant supplementation.

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ABBREVIATIONS

BHT, butylated hydroxytoluene; CD, conjugated diene; IL-2R, interleukin-2 receptor; NF-κB, nuclear factor-κB; TBA, thiobarbituric acid; TBARS, TBA reactive substances; TNFα, tumor necrosis factor-α; VO_{2max}, maximal O_2 consumption.

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