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Exercise elevates plasma levels but not gene expression of IL-1β, IL-6, and TNF-α in blood mononuclear cells

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Moldoveanu, Andrei I., Roy J. Shephard, and Pang N. Shek. Exercise elevates plasma levels but not gene expression of IL-1β, IL-6, and TNF-α in blood mononuclear cells. *J Appl Physiol* 89: 1499–1504, 2000.—Physical activity induces a subclinical inflammatory response, mediated in part by leukocytes, and manifested by elevated concentrations of circulating proinflammatory cytokines, including interleukin (IL)-1β, IL-6, and tumor necrosis factor-α (TNF-α). However, the source of the cytokines that appear during exercise remains unknown. In this study, we examined exercise-induced changes in plasma cytokine concentrations and their corresponding mRNA expression in peripheral blood mononuclear cells. Ten healthy [peak oxygen uptake = 48.8 ± 6.5 (SD) $ml \cdot kg^{-1} \cdot min^{-1}$] but untrained men [age = 25 ± 5 (SD) yr] undertook 3 h of exercise (cycling and inclined walking) at 60–65% peak oxygen uptake. Circulating leukocyte subset counts were elevated during and 2 h postexercise but returned to normal within 24 h. Plasma concentrations of IL-1β, IL-6, and TNF-α peaked at the end of exercise and remained elevated at 2 h (IL-6) and up to 24 h (IL-1β and TNF-α) postexercise. Cytokine gene expression in circulating mononuclear cells was measured by using the reverse transcriptase-polymerase chain reaction; mRNA accumulation did not change with exercise. In conclusion, mRNA accumulation of IL-1β, IL-6, and TNF-α in circulating mononuclear cells is not affected by 3 h of moderate endurance exercise and does not seem to account for the observed increases in plasma cytokines.

physical exertion; immune; messenger RNA; peripheral blood

CYTOKINES ARE GLYCOSYLATED polypeptides that are secreted by, and influence the action of, most cells of the body (34). The proinflammatory cytokines, including interleukin (IL)-1β, IL-6, and tumor necrosis factor-α (TNF-α), modulate immune cell function and migration, initiating and amplifying the acute-phase and stress responses, and pyrogenesis (2, 10, 14). The local production of these molecules coordinates the function of innate and adaptive immune cells, including the interactions with vascular endothelial cells, differential expression of cell-surface effector molecules, growth, and differentiation. In certain pathological states such as trauma, sepsis, and thermal injury, proinflammatory cytokines are released into the circulation (21). The outcome is then dictated not by the nature of the original insult but rather by the manner in which the body regulates and attenuates cytokine production through the action of various immune and nonimmune cells (26). The cytokine response to sepsis, for example, induces peripheral vasodilation, the ensuing organ hypoperfusion, and the multiple-organ failure that are commonly observed. There are many similarities between the pattern of cytokine elaboration brought on by prolonged (1- to 3-h) bouts of physical activity and various forms of pathological insult (6, 8, 27). Superficially, the body reacts to physical activity as it does during an acute, subclinical inflammatory response to a perceived pathological insult (27, 32). Pro- and anti-inflammatory cytokines are released into the circulation; along with other bioactive stress molecules, including glucocorticoids and catecholamines, cytokines regulate various aspects of the immune system.

Patterns of response to sepsis and trauma are well established (16). Both sepsis in vivo and leukocyte stimulation in vitro can activate the pathway of cytokine production, from mRNA expression to protein secretion, in subsequently isolated immune cells. In an intact host, this process can translate into extreme, pathological levels of circulatory cytokines, resulting in immune system activation, suppression, or anergy, depending on the level and profile of secretion (32). In contrast, very little work has focused on determining whether circulating immune cells, known to produce proinflammatory cytokines, are responsible for the apparent mild inflammatory response to exercise (22, 28, 35, 36). The aim of the present study was to determine whether exercise-induced increases in plasma concentrations of IL-1β, IL-6, and TNF-α could be explained by changes in messenger RNA accumulation in peripheral blood mononuclear cells (PBMCs). Such information would be helpful in determining how far exercise

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can be characterized as a model of inflammation, and in establishing the effects of prolonged acute endurance exercise on immune function and cytokine biology.

METHODS

Subjects. Ten healthy [peak oxygen uptake ($\dot{V}O_{2\,peak}$) = 48.8 ± 6.5 (SD) ml·kg^{-1}·min^{-1}] but untrained men [mean age = 25 ± 5 (SD) yr; body mass = 76.2 ± 4.8 (SD) kg; body fat = 15.8 ± 2.9 (SD) %] were recruited from the Defence and Civil Institute of Environmental Medicine, the University of Toronto, and York University. Ethical approval was obtained from the Human Subjects Research Committee of the University of Toronto and from the Defence and Civil Institute of Environmental Medicine, and written, informed consent was obtained from all subjects.

Experimental design and exercise protocol. Subjects reported to the laboratory on four separate occasions. Figure 1 illustrates the exercise protocol and the blood sampling scheme. An initial visit was required for medical screening and anthropometric and physiological measurements. Subjects deemed unfit and/or unhealthy by the medical screening officer were excluded. Criteria for rejection included a body fat >30%, cigarette smoking, symptoms of current illness, or use of anti-inflammatory drugs. $\dot{V}O_{2\,peak}$ was determined by using an incremental graded cycle ergometer test. In a familiarization trial, subjects spent 60 min at 60–65% $\dot{V}O_{2\,peak}$; exercise was divided into 20-min portions on a cycle ergometer, inclined treadmill, and cycle ergometer, all while catheterized in preparation for the full-length 3-h exercise trial.

Subjects performed both an exercise and a control trial at corresponding periods of the day, with randomization of order. The subjects arrived 1 h before the start of exercise. At least 7 days of recuperation were allowed between any of the trials requiring blood sampling or exercise. Subjects fasted for 12 h before exercise, and they were fed at least one, but no more than three servings of meal replacement drink (Ensure Plus, Abbot Laboratories, Saint Laurent, PQ, Canada) in the morning of the trial. Venous catheterization was performed shortly thereafter, and subjects then sat for 30 min until the start of exercise. The exercise trial involved three 60-min exercise bouts, performed without pause or recuperation. One hour on a cycle ergometer was followed by 1 h on an inclined treadmill and then by a further 1 h on a cycle ergometer (Fig. 1). The work rate was individually adjusted and maintained at 60–65% of peak aerobic power as determined on the cycle ergometer. Blood was sampled (19-ml aliquots) at selected times throughout exercise and for 2 h subsequently. Subjects returned 24 h postexercise for a final blood draw.

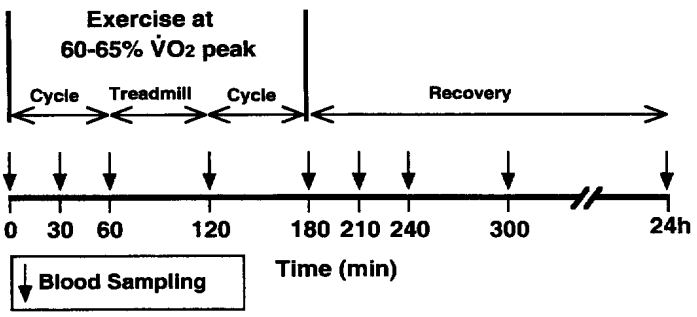

Fig. 1. Exercise protocol and blood sampling scheme. $\dot{V}O_{2\,peak}$, peak oxygen uptake.

Physical and physiological measurements. Peak oxygen intake was determined on a cycle ergometer (model 816E Monarch) after a graded protocol to subjective exhaustion. Respiratory gases were analyzed by using a Sensor Medics MMC Horizon System 4400 metabolic cart (Anaheim, CA). Subjects maintained a cadence of 70 pedal revolutions/min throughout. During both maximal exercise testing and the prolonged exercise trials, heart rate was monitored continuously via a transmitter-telemetry unit (Polar Vantage XL, Polar CIC, Port Washington, NY). Height and body mass were measured at the beginning of the peak exercise day, as well as on the days of the exercise and the control trials.

Blood sampling and hematology. On days that required multiple blood sampling, an intravenous catheter was inserted into a prominent vein in the antecubital fossa; if a single blood sample was needed, a standard venipuncture was performed. Total white cell count, differential leukocyte counts (granulocytes, monocytes, lymphocytes), hemoglobin, and hematocrit were determined, by using a Coulter JT automatic hematology system (Coulter Electronics, Hialeah, FL). Adjustment was made for physical activity-induced blood volume and plasma volume changes, by using the method of Dill and Costill (13).

PBMC isolation. Blood was drawn into 10-ml glass vacuum tubes (Vacutainer systems, Becton Dickinson, Franklin Lakes, NJ) containing 72 USP units of sodium heparin. Blood samples were diluted with an equal volume of PBS (Sigma-Aldrich Canada, Oakville, ON, Canada), and PBMCs were isolated by centrifugation through Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden). For each sample, two 15-ml centrifuge tubes were used to layer 7 ml of diluted blood onto an equal volume of Ficoll-Hypaque. The suspension was centrifuged for 30 min at 450 *g* and 20°C. The mononuclear cell layer was removed with manual pipetting, washed twice with PBS, and centrifuged for 10 min at 10°C and 275 *g* after each wash. Washed cells were resuspended in 1 ml of PBS. The total leukocyte count was determined by an electronic cell counter (model ZM Coulter counter, Luton, Beds, UK), and the volume was then adjusted to 2×10^6 cells/ml with PBS.

Isolation of total ribonucleic acid. Immediately on isolation and dilution to 2×10^6 cells/ml, the PBMC suspension was centrifuged for 3 min at 3,000 *g*, and the supernatant was discarded. Total RNA was isolated by using the Totally RNA isolation kit (Ambion, Austin, TX). Briefly, cells were lysed in a guanidinium-based denaturation solution by using 167 μl of denaturation solution per 10^6 cells. Samples were either processed immediately or stored at −70°C for a maximum of 2 mo. Two phenol-chloroform extractions of increasing acidity and ionic conditions were performed, after which the RNA was precipitated through 100% isopropanol incubation at −20°C for 90 min, with subsequent centrifugation and resolubilization in 15 μl of double-distilled water. An internal control for RNA recovery was not used and an assumption was made on the established reliability and reproducibility of the commercial RNA isolation kit. An indirect indication of the reproducibility of RNA recovery was provided by a relatively consistent expression of the housekeeping glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene and also the IL-1β, IL-6, and TNF-α genes (coefficient of variation: 18.1, 3.2, and 15.2%, respectively; n = 10) in separate experiments.

The concentration and purity of the recovered RNA were established by measuring ultraviolet absorbance at 260 and 280 nm (model DU-50 spectrophotometer, Beckman Instruments, Fullerton, CA). The intactness of the RNA was determined by electrophoresing a small fraction of the sample in a

1.5% (wt/vol) high-resolution nondenaturing agarose gel (Sigma-Aldrich Canada) stained with ethidium bromide. The presence of nondegraded eukaryotic 18S and 28S ribosomal RNA bands was diagnostic of intact total RNA.

Reverse transcription. Polyadenylated RNA message was reverse transcribed by using the RetroScript kit (Ambion). In summary, 2 μg of total RNA were dissolved in 10 μl of double-distilled water, to which was added 4 μl of dNTP mix (2.5 mM each of dATP, dGTP, dCTP, dTTP) and 2 μl of 50 mM first-strand primer [oligo$(dT)_{18}$]. After gentle mixing, the reaction tube was heated to 75–80°C for 3 min to relieve RNA secondary structure, and the tube was then immediately placed on ice to promote primer hybridization. Two microliters of 10× reverse transcriptase-polymerase chain reaction buffer (100 mM Tris·HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$), 1 μl of placental RNase inhibitor (10 U/μl), and 1 μl of murine-Maloney leukemia virus (M-MLV) enzyme (100 U/μl) were added, and the reaction was mixed, recollected by centrifugation, and incubated at 42°C for 90 min. The M-MLV reverse transcriptase enzyme was subsequently inactivated by denaturation in a water bath for 10 min at 92–95°C.

Competitive, quantitative polymerase chain reaction. Amplification of IL-1β, TNF-α, and IL-6 RNA message cDNA was performed by using the CytoXpress kit (Biosource International, Camarillo, CA). In brief, 2 μl of reverse transcription reaction mixture and 5 μl (2×10^3 copies) of internal calibration standard (400 copies/μl) acted as templates. To attain a final reaction volume of 100 μl, the following reagents were added: 10 μl of 10× polymerase chain reaction buffer (100 mM Tris·HCl; pH 8.3, 500 mM KCl, 15 mM $MgCl_2$), 8 μl dNTP mix (2.5 mM each dNTP), 2 μl cytokine-specific primer pair (25 pmol/μl), 0.5 μl of SuperTaq thermostable DNA polymerase (5 U/μl; Ambion), and 72.5 μl of double-distilled water. After an initial denaturation for 2.5 min at 95°C, the samples were thermally cycled (GeneAmp PCR System 2400, Perkin Elmer, Norwalk, CT) for 30 s at 95°C, 45 s at 55°C, and 1 min at 72°C for 35 cycles. Amplified DNA was stored at −20°C for a maximum of 2 wk, until quantified.

To demonstrate the ability of reverse transcriptase-polymerase chain reaction to quantify changes in gene expression, the effects of lipopolysaccharides (*Escherichia coli* serotype 026:B6, Sigma-Aldrich Canada) stimulation (10 μg/ml of whole blood, 37°C, 4 h) on the accumulation of polyadenylated RNA of IL-1β, IL-6, TNF-α, and GAPDH in PBMCs of three subjects were assessed in a separate experiment. Whereas the messenger RNA copy number of GAPDH was unchanged by lipopolysacchardide stimulation, cytokine message values increased 93-, 87-, and 96-fold for IL-1β, IL-6, and TNF-α, respectively.

Agarose gel DNA band densitometry quantification. DNA was separated according to size on a 1.5% high-resolution native agarose gel (Sigma-Aldrich Canada) at 100 V and 90 mA for ~3 h. The gel was visualized under ultraviolet light (312 nm) and photographed. The gel image was digitized, and the band intensity was quantified by using the gel-analysis software UN-Scan-It Gel version 3.1 (Silk Scientific, Orem, UT), with preprogrammed algorithms. The messenger RNA copy number was backcalculated by comparing the cytokine message band intensity to that of the internal calibration standard, which was administered at a known copy number of 2×10^3.

Plasma cytokine measurement. Venous blood was collected in pyrogen-free vacutainers (Vacutainer systems, Becton Dickinson) containing K_3EDTA (1.44 mg/5 ml blood). Blood samples were immediately centrifuged (1,000 *g*, 15 min, 4°C), and the supernatant was harvested and stored at −70°C until processed further. Plasma concentrations of IL-1β, IL-6, and TNF-α were assayed by using the quantitative high-sensitivity ELISA technique in kit form according to the supplier's instructions (R&D Systems, Minneapolis, MN). The absorbance of the product (measured in optical density units), determined with an automated spectrophotometer-microtiter plate reader (model EL-340, Bio-Tek Instruments, Winooski, VT), was directly proportional to the amount of cytokine in the standard or sample. Absorbance was converted to concentration (in pg/ml), by using standard curves.

Statistical analyses. Hematologic and messenger RNA data were analyzed by using a two-factor (time and condition) repeated-measures ANOVA. Data on circulating cytokines were analyzed using a one-factor model (time) repeated-measures ANOVA. On finding a significant F ratio for dependent interaction measures, main effects were tested by using the least squares method of contrasts. A P value ≤ 5% was accepted as significant.

RESULTS

Leukocyte subsets. The effects of 3 h of 60–65% $\dot{V}O_{2\,peak}$ exercise on total leukocyte, granulocyte, lymphocyte, and monocyte counts are illustrated in Fig. 2. Total leukocyte counts were increased significantly within 30 min of the commencement of exercise, but it did not peak until the conclusion of physical activity at 3 h, attaining a cell count of 21.2×10^9/l. Throughout the 2-h recovery period, the total white cell count remained above control values, tending to stabilize at $\sim 15 \times 10^9$/l, and returning to normal at 24 h.

The prolonged exercise bout induced a pronounced granulocytosis, which was largely responsible for the changes in total white cell count. Significant increases in circulating granulocyte counts began within 30 min after the start of exercise, but, like total leukocytes, the cell count did not peak until the end of exercise. Values remained significantly elevated ($P < 0.05$) for at least 2 h postexercise but had returned to baseline at the 24-h sampling time point.

Circulating total lymphocyte count increased within 30 min from the onset of exercise and peaked at the end of exercise, at 4.3×10^9/l, with a return to normal after exercise.

The circulating monocyte count rose significantly within 30 min of the commencement of exercise, to 0.5×10^9/l, but did not peak until the end of exercise, when values reached 1.0×10^9/l. Cessation of exercise was associated with a sharp initial drop in circulating monocyte count to near control values, followed by a significant rebound for the remainder of the 2-h recovery. At 24 h, monocyte counts returned to normal.

Plasma cytokine response. Plasma cytokine levels of IL-1β, IL-6, and TNF-α were measured in six exercising subjects at various time points from the beginning of the exercise bout (Fig. 3). An initial plasma IL-1β concentration of 0.04 pg/ml had increased to 0.19 and 0.59 pg/ml by 60 and 180 min into exercise, respectively ($P < 0.05$). Within 2 h after the end of exercise, IL-1β concentration had fallen to 0.20 pg/ml, although concentrations were still significantly higher than pre-exercise concentrations both at 300-min and 24-h time points.

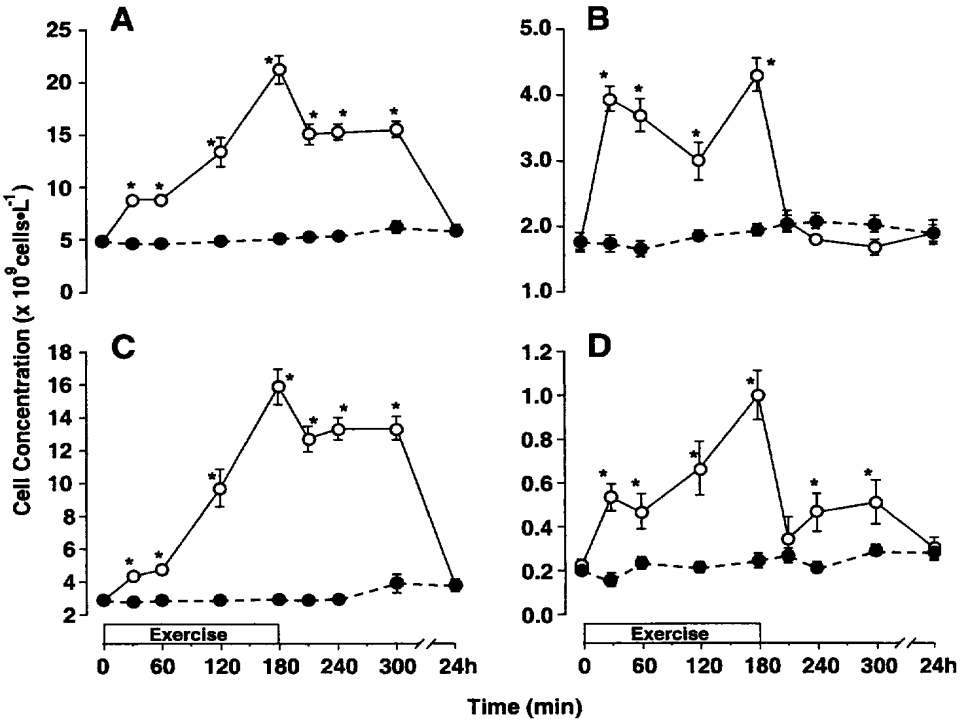

Fig. 2. Leukocyte (*A*), lymphocyte (*B*), granulocyte (*C*), and monocyte (*D*) counts in control (●) and exercise (○) trials. Values are means ± SE for 10 subjects. *$P <$ 0.05 relative to control values.

IL-6 increased from a preexercise concentration of 1.2 to 4.8 pg/ml after 60 min of exercise ($P < 0.05$) and peaked (21.8 pg/ml) at the end of exercise. Plasma IL-6 concentration was still significantly elevated 2 h into recovery and had returned to baseline levels by the 24-h sampling time point.

An initial plasma TNF-α concentration of 1.0 pg/ml increased to 1.2 and 1.9 pg/ml after 60 and 180 min of physical activity, respectively ($P < 0.05$). Concentrations had decreased at the 300-min and 24-h time points, although values were still significantly greater than at initial rest.

Cytokine messenger RNA response. Accumulation of mRNA for IL-1β, IL-6, and TNF-α was measured in PBMCs of subjects in both control and exercise trials at all time-points. Messenger RNA accumulation of IL-1β in both control and exercising subjects remained unchanged, relative to their respective repeated measure, in both control and exercise trials (Fig. 3). Messenger RNA copy numbers accumulated in PBMCs ranged from 2.1 to 2.6 × 10^4/μg total RNA. IL-6 copy number per microgram of total RNA ranged from 1.4 to 2.1 × 10^3. Again, no significant single time-point differences between conditions were noted. Similarly, exercise had no detectable effect on the gene expression of TNF-α in PBMC, the detectable accumulation of TNF-α mRNA/μg total RNA ranging from ~8.0–10.0 × 10^3 in both control and exercising subjects.

DISCUSSION

In agreement with other studies (1, 31), prolonged endurance exercise induced a transient elevation in circulating leukocyte counts, driven largely by a granulocytosis but also influenced by an increase in monocytes and lymphocytes. Nevertheless, the accumulation of mRNA specific for IL-1β, IL-6, and TNF-α in circulating mononuclear cells seems unlikely to account for the increased plasma concentrations of these proinflammatory cytokines during and immediately after exercise.

The stimuli leading to the increase in various leukocyte subsets during exercise are well understood. The effects of hemoconcentration are thought to be small (15, 19). Contraction of the spleen and other secondary lymphoid organs accounts for a small, but possibly an important, fraction of the commonly observed increase in circulating immunocytes, particularly that of lymphocytes (19). Altered hemodynamics do not explain all of the observed changes (25). Changes in plasma concentrations of catecholamines and glucocorticoids potently modulate immune cell migration and activity during exercise (17). Catecholamine-induced changes in the interaction between lymphocytes and vascular endothelial cells are thought to increase circulating counts through a rapid demargination of immune cells (19). Depending on the levels of carbohydrate stores and of psychophysiological stress, glucocorticoids are released during and after prolonged endurance exercise (24). Cortisol may counter the effects of β-adrenoreceptor stimulation on circulating lymphocyte counts, inhibiting their entry into the circulation and promoting their exit into peripheral tissues (11).

We searched for a possible relationship between exercise-induced elevation in specific plasma cytokine levels and corresponding changes in messenger RNA accumulation in PBMCs. The peak IL-6 level was significantly

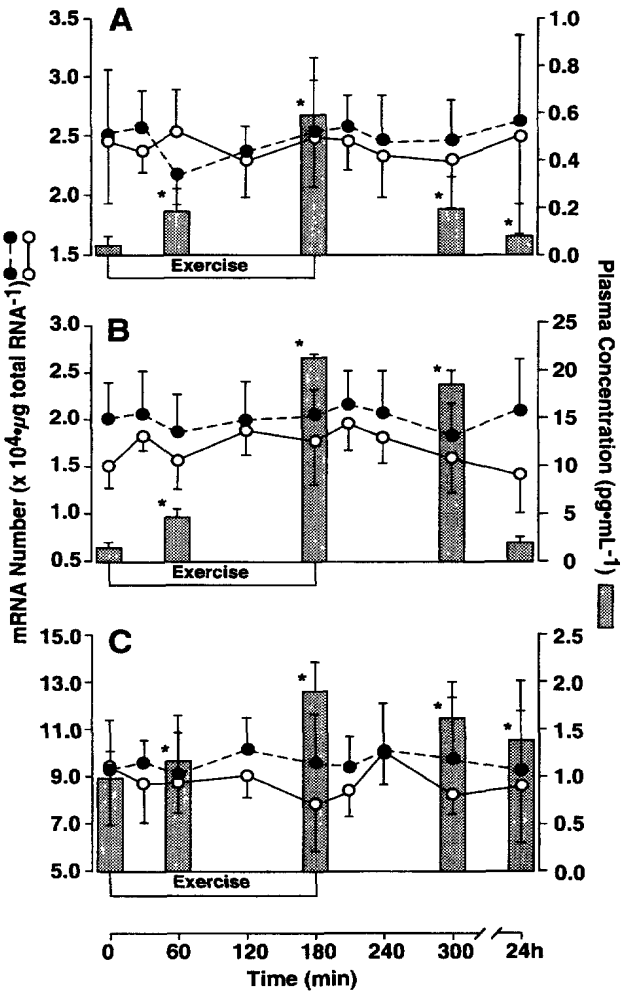

Fig. 3. Plasma concentrations of interleukin-1β (*A*), interleukin-6 (*B*), and tumor necrosis factor-α (*C*) at various times during exercise (vertical bars), with peripheral blood mononuclear cell mRNA accumulation of interleukin-1β, interleukin-6, and tumor necrosis factor-α in control (●) and exercise (○) trials. Values are means ± SE for 10 subjects. *$P < 0.05$ relative to preexercise values.

elevated by ~17-fold, but the elevated concentration was only about one-third of that typically seen after a competitive marathon race. The PBMC gene expression of IL-6 did not change relative to control trials and is thus unlikely to account for changes in plasma IL-6 concentrations. Discounting the uncommon circumstances in which cytokines are produced and stored by immune cells until subsequent release (33), it seems likely that the increased plasma concentrations of IL-6 were secondary to genetic upregulation in cells other than PBMC, for instance myocytes, macrophages or fibroblasts. Other investigators have shown that IL-6 mRNA accumulates in muscle biopsy samples after exercise (28), but exhausting exercise does not change the levels of IL-6 gene expression in spleen cells (36), lending support to our conclusion. In contrast, IL-6 gene expression in PBMCs is strongly upregulated by septic infection, where accumulation of messenger RNA correlates closely with elevated plasma concentrations (7, 12). In trauma, changes in plasma IL-6 are not always secondary to changes in IL-6 transcriptional events in PBMCs (18). IL-6 production is intimately related to tissue damage (5), helping to recruit circulating monocytes and neutrophils to damaged tissues to expedite healing and scar formation (20). Possibly, IL-6 plays a similar role in exercise-induced muscle damage, although the response is smaller because of a lesser degree of injury. If so, IL-6 may be produced and released by cells intrinsic to the affected area, be these integral tissue cells proper or tissue macrophages. However, it is also possible that our observed increase in circulating IL-6 could be attributable, at least in part, to a corresponding elevation in circulating monocyte and lymphocyte counts.

The present findings do not resolve the cause or biological significance of the increased circulating concentrations of IL-1β and TNF-α during and after exercise. Strenuous exercise-induced elevations in IL-1β and TNF-α have been reported, but results from different studies appeared inconsistent, possibly in part due to differences in experimental design, timing of blood sampling, and cytokine assay sensitivity (32). Exhausting exercise can augment blood levels of endotoxin, presumably through increases in gastrointestinal permeability (4, 8); if this is the stimulus for the production of proinflammatory cytokines, it is likely that circulating monocytes are responsible, as in the case of pathological endotoxemia (29). However, we noted no change in gene expression in circulating mononuclear cells, making this an unlikely mechanism in the present study. Cytokine elaboration is certainly among the effector functions of activated immune cells, but because monocytes and lymphocytes circulate in a mature but inactivated state, it seems unlikely that the circulating cells would be responsible for a rapid increase in plasma cytokine concentrations. Primary and secondary lymphoid organ leukocytes outnumber PBMC by 10- to 100-fold, and it seems possible that these cells respond to a mild exercise-induced endotoxemia by producing and releasing TNF-α and IL-1β.

Although 3 h of exercise at 60–65% $\dot{V}O_{2\,peak}$ is a significant and unaccustomed physical challenge for recreationally active, untrained subjects, the possibility remains that the exercise protocol we adopted was an insufficient physiological stressor to modify cytokine gene expression in circulating PBMCs. It would be instructive to measure changes in plasma cortisol and endotoxin concentrations in future experiments. Furthermore, cycle ergometry and inclined treadmill are both largely concentric activities, and it would be interesting to repeat observations after eccentric exercise, when muscle damage is likely to be greater.

Blood levels of cytokines represent the net effect of genetic upregulation, catabolism in the blood and tissues, binding to soluble receptors, biological use by various cells, and removal from the body via liver metabolism and renal excretion (3, 30). The lag time that exists between the onset of gene expression and detectable changes in the blood varies greatly (23). Data from the present study must be interpreted in light of this variability. Nevertheless, several lines of evidence from other investigations

support our view that cells, other than circulating monocytes and lymphocytes, could be responsible for the increases of plasma cytokine concentration during and after exercise.

In conclusion, prolonged endurance exercise elevates plasma concentrations of IL-1β, IL-6, and TNF-α, but it does not change the accumulation of the corresponding cytokine messenger RNAs in PBMCs. Although our observations do not preclude the involvement of PBMCs in the increased plasma concentration of proinflammatory cytokines during exercise, a contribution by PBMCs at the level of gene expression seems unlikely. It remains possible that circulating immunocytes are releasing presynthesized intracellular cytokines in response to exercise. However, this seems unlikely to be the case, given that cytokines are commonly regulated pretranscriptionally and that cells other than circulating immunocytes (9, 28) contribute to cytokine production during exercise.

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