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Endurance run increases circulating IL-6 and IL-1ra but downregulates ex vivo TNF- α and IL-1 β production

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Drenth, Joost P. H., Stan H. M. Van Uum, Marcel Van Deuren, Gerard J. Pesman, Johanna Van Der Ven-Jongekrijg, and Jos W. M. Van Der Meer. Endurance run increases circulating IL-6 and IL-1ra but downregulates ex vivo TNF- α and IL-1 β production. *J. Appl. Physiol.* 79(5): 1497–1503, 1995.—We investigated whether a 6-h endurance run changes cytokine plasma concentrations and lipopolysaccharides (LPS) stimulated ex vivo production of cytokines in a whole blood culture of 19 well-trained athletes. The average distance covered was 65.1 ± 8.64 (SD) km. At the end of the exercise, the mean plasma concentration of interleukin-1-receptor agonist (IL-1ra), which was 188 pg/ml 24 h before finish, increased to 886 pg/ml ($P < 0.0005$). The mean plasma interleukin-6 concentration increased from 18.5 ± 4.2 to 71.5 ± 33.3 pg/ml ($P < 0.0001$). The increase of neutrophils correlated with the increase of IL-1ra concentrations ($r = 0.58$, $P < 0.005$). We could not detect an effect of exercise on plasma concentrations of interleukin-1 β (IL-1 β) or tumor necrosis factor- α (TNF- α). The ex vivo LPS-stimulated production of IL-1 β in athletes 24 h before the run was significantly higher than in sedentary controls. Exercise induced a decrease of LPS-stimulated production of IL-1 β and TNF- α , whereas production of IL-1ra was unchanged. These results show that prolonged exercise elicits a selective downregulation of the proinflammatory cytokine production and upregulation of the cytokines IL-1ra and interleukin-6.

endurance run; cytokines; cytokine production; inflammation

PROINFLAMMATORY CYTOKINES such as tumor necrosis factor- α (TNF- α), interleukin (IL)-1, IL-6, and the anti-inflammatory IL-1-receptor antagonist (IL-1ra) are involved in various immunologic and inflammatory processes (38). In this respect, there are indications that physical exercise modifies plasma cytokine concentration and its production. Early studies indicated that plasma IL-1 bioactivity increases after exercise (6), but this was not confirmed in later studies using immunoassays (29, 33, 36). For example, a 20-km run did not influence the plasma immunoreactive concentrations of IL-1 β , TNF- α , and IL-6 in well-trained runners, although the urinary secretion of these cytokines was temporarily increased just after exercise (33). The effect of physical exercise on ex vivo production of cytokines by blood cells has not been studied extensively, and results are rather conflicting. When using isolated monocytes, Rivier et al. (30) noted a modest increase of spontaneous TNF- α production after exercise, whereas Haahr et al. (20) found no such difference after lipopolysaccharide (LPS) stimulation.

We therefore decided to study the effect of intense prolonged exercise (6-h run) on plasma concentrations

of IL-1 β , IL-6, TNF- α , and IL-1ra and ex vivo production (using a whole blood culture system) of IL-1 β , TNF- α , and IL-1ra.

MATERIALS AND METHODS

Exercise protocol and human subjects. A total of 73 athletes (71 men and 2 women) entered a 6-h endurance run competition on October 16, 1994, in Geldrop, The Netherlands. Thirty participants were asked by telephone, in the weeks before the run, to join the proposed study. Finally, 21 well-trained long-distance male athletes (mean age 43.7 ± 8.4 yr, range 26–59 yr) participated in our study. These volunteers are regularly involved in long-distance running competitions. The ambient temperature at that day was 12°C, with a humidity of 70%. The wind velocity was 5.5 m/s. After explanation of the aims of the study, written informed consent was obtained from all participants. The participants refrained from exercise in the 24-h period before the first test. The study was approved by the Ethical Committee of the St. Joseph Hospital in Veldhoven, The Netherlands.

Blood samples. Blood samples were drawn from the antecubital vein at rest in a sitting position 24 h before the projected end of the run (to correct as much as possible for circadian effects) and immediately after end of the exercise. Blood for cytokine measurements was collected into 4-ml (circulating) and 2-ml (ex vivo production) endotoxin-free EDTA tubes (Vacutainer Systems, Becton and Dickinson, Rutherford, NJ). The plasma was immediately processed to avoid ex vivo cytokine production and release (11). Cytokine production was measured using a whole blood culture system as described elsewhere (39). Briefly, two 2-ml tubes containing 24 μ l EDTA-K₃ (10,000 kallikrein-inactivating units/ml; Bayer, Leverkusen, Germany) were drawn.

One tube was incubated immediately, the other was incubated after addition of 25 μ l LPS (*Escherichia coli* serotype 055:B5; Sigma Chemical, St Louis, MO; final concentration 10 μ g/ml blood). After 24 h of incubation at 37°C, both tubes were centrifuged at 2,250 g for 10 min and then at 15,000 g for 5 min to obtain platelet-poor plasma. Aliquots were stored at -70°C until assay. Hemoglobin, leukocyte numbers, and differential counts before and after the run were determined with an automated Coulter counter STKS (Coulter Electronics Hialeah FL).

Source of antiserum. Polyclonal antibodies for IL-1 β were kindly provided by Scavo (Siena, Italy), and antibodies for TNF- α were a gift of Dr. C. A. Dinarello (New England Medical Center, Boston, MA). A polyclonal anti-IL-1ra antiserum was raised in New Zealand White rabbits immunized with human recombinant IL-1ra kindly provided by Synergen (Boulder, CO).

Radiolabeling of cytokines. Human recombinant IL-1 β and TNF- α were radiolabeled by using the chloramine-T method (37). Briefly, 5 μ g IL-1 β or TNF- α in 10 μ l of 0.5 M phosphate buffer, pH 7.4, and 18.5 MBq Na¹²⁵I (Amersham Interna-

tional, UK) and 4 μl chloramine T (8.9 mmol/l) were added to the tubes and incubated for 10 s. The reaction was terminated by adding 20 μl $\text{Na}_2\text{S}_2\text{O}_5$ (26.3 mmol/l) in 0.25 M phosphate buffer.

Human recombinant IL-1ra was radiolabeled with ^{125}I by the method of Bolton and Hunter (5). In short, 0.28 μg of Bolton-Hunter reagent (0.1 $\mu\text{g}/\mu\text{l}$) was radioiodinated with 37 MBq ^{125}I by adding 5 μg NCS (1 $\mu\text{g}/\mu\text{l}$) as a reductor during 10 min at room temperature. The reaction was stopped after the addition of 5 μg $\text{Na}_2\text{S}_2\text{O}_5$ as an oxidator. The mixture was evaporated to dryness during ~ 1 h under a stream of N_2 , and subsequently 4 μg IL-1ra (5 $\mu\text{g}/\mu\text{l}$) in 1.0 M bicarbonate buffer, pH 8.2, were added and incubated for 15 min at 0°C . After the labeling reactions, the reaction mixtures were applied to a Sephadex G-25 column (PD-10; Pharmacia, Uppsala, Sweden) and eluted with phosphate-buffered saline (PBS) (IL-1 β and TNF- α) or 0.25% gelatine in PBS (IL-1ra) to separate labeled proteins from free ^{125}I .

Radioimmunoassay (RIA) for cytokines. IL-1 β , TNF- α , and IL-1ra in plasma were measured in duplicate by nonequilibrium RIA. Recombinant human IL-1 β , TNF- α , and IL-1ra were calibrated against standards provided by the National Institute of Biological Standards and Control (Potters Bar, UK). The RIA was performed as follows. One hundred microliters of rabbit polyclonal cytokine antibodies (final dilution of IL-1 β 1:210,000; TNF- α 1:150,000; and IL-1ra 1:60,000, giving a binding with tracer of $\sim 25\%$), dissolved in RIA buffer per liter containing 13 mM Na_2EDTA , 0.02% sodium azide, 0.25% bovine serum albumin (Behring, Marburg, Germany), 0.1% Triton X-100 3 mM $\text{Na}_2\text{H}\cdot\text{PO}_4$, and 250,000 kallikrein-inactivating units aprotinine, pH 7.4 (Bayer, Leverkusen, Germany), were added to sample and standard. All reagents were prepared with this buffer. For circulating cytokine measurement, 100 μl of sample and standard were added, and for measurement of ex vivo cytokine production, 25 μl (TNF- α) and 10 μl (IL-1 β and IL-1ra) of sample and standard were added. The mixture was incubated for 1 day at room temperature. Subsequently, tracer ($\approx 7,000$ dpm/100 μl) was added, and incubation was continued for another 2 days.

To separate bound and free tracer from the free fraction, 100 μl of a separation agent [10% (vol/vol) sheep anti-rabbit immunoglobulin G and 0.01% (wt/vol) rabbit immunoglobulin G (Sigma, St. Louis, MO)] were added to each tube and incubated for 30 min at room temperature. The antibody complex was completely precipitated by the addition of 1 ml 7.5% polyethylene glycol 6000 (Merck, Darmstadt, Germany). For IL-1 β , TNF- α , and IL-1ra, the range of the standard curve was 20–3,000 pg/ml. The sensitivity of the assay with 100- μl sample was 60 pg/ml (IL-1ra), 40 pg/ml (IL-1 β), and 20 pg/ml (TNF- α). To minimize analytic errors, all samples from the same patient were analyzed in the same run in duplicate.

In our assay for IL-1 β , with complete specific precipitation of the first antibody with the second antibody, chloroform extraction for eliminating interfering factors is not needed (9). Samples treated with chloroform extraction showed a good correlation to those without treatment ($r = 0.77$). The interference of soluble interleukin-1 receptors (sILr) with the measurement of IL-1 β has not actually been tested in this assay. The recovery, however, of IL-1 β with this assay is 70%. The linearity, as tested by addition of two samples with unknown IL-1 β content to a sample with established IL-1 β concentration, gave a correlation coefficient of 0.99. A fine parallelism has been shown for IL-1 β ($r = 0.90$) for sample volumes from 3 up to 100 μl . For TNF- α , this RIA measures total TNF- α (both free and complexed to its receptors), as demonstrated by the lack of interference of the addition up to 5 ng/ml recombinant soluble tumor necrosis factor receptors

(sTNFr) (p55 and p75) to sera containing known amounts of TNF- α (4). The recovery of TNF- α in our RIA was estimated at 93.3%. However, no satisfying parallelism was achieved with TNF- α with this assay. To avoid this problem, sample volumes were adjusted for measurement of circulating TNF- α (100 μl) and ex vivo production (25 μl), and with these quantities accurate intragroup comparison of results was possible.

For IL-1ra, the recovery was estimated at 86.2%. Good parallelism is achieved with native human IL-1ra reagents. With human recombinant IL-1ra, complete parallelism is lacking and only measurements from fixed amounts of sample can be compared. Therefore, the sample volume for measurement of circulating IL-1ra is 100 μl , compared with 10 μl for assessment of ex vivo production. To correct for spontaneous ex vivo cytokine production, we calculated the net LPS-stimulated production according to the following formula: net cytokine production = production with LPS – production without LPS.

The interassay variation of our RIA is estimated at $< 15\%$, whereas the intra-assay variation is $< 10\%$, which are typical results for these assays.

Control values for cytokine ex vivo production were obtained at a given day from 10 healthy sedentary volunteers (20–45 yr of age). These values were $6,847 \pm 2,175$ pg/ml (IL-1 β); $3,344 \pm 969$ pg/ml (TNF- α), and $9,827 \pm 2,597$ pg/ml (IL-1ra). Very similar concentrations were found in samples drawn from the same volunteers 24 h later.

Enzyme-linked immunosorbent assay (ELISA) for IL-6. IL-6 was measured with an ELISA (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, the Netherlands) as follows (21). Flat-bottom microtiter plates were coated for 24 h at room temperature with anti-IL-6 monoclonal antibody (diluted 1:100 in coating buffer). The plates were washed five times with PBS containing 0.005% Tween 20 and were incubated for 1 h with PBS containing 5% bovine serum albumin (Sigma Chemical A.7030) to prevent aspecific binding. Subsequently, the plates were washed five times with washing buffer, and samples (100 μl) were added.

After incubation for 1 h at 37°C , the plate was again washed five times, and biotinylated sheep antibody to human IL-6 in conjugate buffer (100 μl) was added to the wells and incubated for 1 h longer at 37°C . After five more washes, streptavidin horseradish peroxidase conjugate (diluted 1:10,000) was added and the plate was incubated for 30 min at room temperature. After the samples were washed again five times, 100 μl of substrate (2,2-amino-bis C3-ethylbenz-thiazoline sulfonic acid, pH 4.0) were added, and after 30-min incubation at room temperature the color reaction was terminated with 100 μl 2% oxalic acid. The optical density was read at 450 nm with a Titertek Multiskan ELISA reader (Eflab, Oy, Helsinki, Finland). The sample values were compared with a standard curve; the lower limit of detection with this assay is 14 pg/ml. There is no interference of sIL-6r in the IL-6 ELISA.

Statistical analysis. The paired nonparametric Wilcoxon signed-rank test was used for statistical comparison of values obtained before and after physical exercise. Probability (P) values were calculated on the basis of two-tailed tests. A correlation coefficient was calculated with the Pearson's correlation test. A $P < 0.05$ was considered to be the lowest level of significance. Data are given in mean \pm SD.

RESULTS

Exercise. The average distance covered by the athletes was 65.1 ± 8.6 km (range 51.7–86.2 km). Two

TABLE 1. Total leukocytes and differential leukocyte count

Parameter	Preexercise	Postexercise
Total leukocytes	7.3±1.6	16.7±4.1
Neutrophils	4.4±1.3	14.5±3.9
Monocytes	0.55±0.1	0.98±0.4
Lymphocytes	2.1±0.6	1.1±0.3

Values are means \pm SD, expressed in $\times 10^9/l$; $n = 19$ for every group. Postexercise values differ significantly ($P < 0.0005$) from pre-exercise values for each parameter listed.

athletes did not complete the 6-h run because of minor injuries. They were excluded from the analysis.

Blood cells. The 6-h endurance run resulted in a significant ($P < 0.0005$) increase in total leukocytes (2.3-fold), neutrophils (3.4-fold), and monocytes (1.8-fold) (Table 1). The lymphocyte count was reciprocally decreased ($P < 0.0005$). There was a moderate positive correlation between covered distance and leukocytosis ($r = 0.47$, $P < 0.05$).

Circulating cytokines. As shown in Figure 1, A and B, there was no effect of exercise on the plasma concentrations of IL-1 β and TNF- α . The plasma IL-1ra concentration increased fivefold during exercise, compared with preexercise values, from 188 ± 72 to 886 ± 395 pg/ml (median 855 pg/ml, range 290–1,450 pg/ml) ($P < 0.0005$) (Fig. 1C). The mean plasma IL-6 concentration increased from 18.5 ± 4.2 to 71.5 ± 33.3 pg/ml (median 64 pg/ml, range 24–130 pg/ml) immediately after the 6-h run ($P < 0.0001$) (Fig. 1D). A positive correlation was observed between the postexercise plasma concentrations of IL-1ra and TNF- α ($r = 0.57$, $P < 0.0005$), of IL-1ra and IL-6 ($r = 0.61$, $P < 0.005$), and of IL-6 and TNF- α ($r = 0.48$, $P < 0.05$). A correlation between postexercise values of IL-1 β and IL-1ra was absent. The increase of neutrophils did correlate with the increase of IL-1ra concentrations ($r = 0.58$, $P < 0.005$).

Ex vivo production of cytokines. Before exercise, ex vivo LPS-stimulated netto production of IL-1 β in whole blood culture from athletes was significantly higher than that of sedentary controls ($P < 0.0005$) (Fig. 2). The preexercise LPS-stimulated production of TNF- α and IL-1ra did not differ between athletes and controls.

Before the run, only for TNF- α a moderate positive correlation of age with LPS-stimulated production could be detected ($r = 0.045$, $P = 0.025$). This positive correlation disappeared after the endurance run.

After exercise, the LPS-stimulated production of TNF- α and IL-1 β decreased appreciably ($P < 0.0005$) (Fig. 2). The IL-1ra production did not change significantly. The LPS-stimulated production of TNF- α correlated with that of IL-1 β ($r = 0.89$, $P < 0.0001$).

DISCUSSION

This study shows that a 6-h endurance run leads to a fivefold increase of plasma IL-1ra concentrations and an almost fourfold increase of IL-6 concentrations in very well-trained individuals, which suggests that prolonged exercise leads to an inflammatory reaction in

humans. IL-1ra is a naturally occurring inhibitor of IL-1 and is able to block its biological activities (14). IL-1ra is synthesized in humans in response to a variety of inflammatory stimuli and appears to limit the deleterious effects of IL-1. In vitro, a number of stimuli appear to induce mononuclear cells to produce IL-1ra in great excess over IL-1 α and IL-1 β . After experimental endotoxin challenge and during infection, large amounts of IL-1ra are found in the circulation compared with only minor quantities of IL-1 (18, 19, 24). It has been established that strenuous exercise leads to an acute phase response with neutrophilia, monocytosis, and elevation of acute phase proteins such as C-reactive protein and fibrinogen (22, 41). IL-1 β , TNF- α , and IL-6 are regarded as major inducers of this response in humans (2). In our study, plasma concentrations of TNF- α and IL-1 β did not change in relation to exercise but we found significant elevations of IL-6 concentrations. These findings are in accordance with other studies in which increased IL-6 concentrations could be measured after prolonged duration exercise (28, 33, 36). There are indications that the kinetics of IL-6 depend on the duration of exercise, since normalization of IL-6 plasma values occurred at 2 h after a 1-h ergometer cycle test at 75% of maximal O₂ uptake but only at 24 h after a 20-km run, which took some 110 min (33, 36). Our finding of unchanged TNF concentrations is in line with those from several investigators (30, 32, 33, 36). In contrast with our results, however, other have reported increased TNF- α concentrations in well-conditioned runners after a 5-km run (16) and after a 2.5-h run (15). One explanation for this discrepancy could be that we missed an increase of TNF- α concentrations because we sampled immediately after the exercise, whereas Dufaux and Order (15) and Espersen et al. (16) detected significant increases of TNF- α at, respectively, 1 and 2 h after the end of the exercise. These investigators did not detect significant increases immediately after the run either (15, 16). A second explanation for this discrepancy is that the increase of TNF concentration is rather short lived, like in experimental endotoxin challenge, and occurred earlier. Therefore, we might have missed the TNF- α elevations. Our inability to detect an increase of TNF- α does not seem to be due to counterregulation by shedding of TNF receptors in the blood, since our RIA detects both free and soluble receptor-bound TNF (4). It is also unlikely that our assay is too insensitive to detect a relevant TNF- α peak, because we have performed control procedures as illustrated by the adequate recovery of exogenous cytokines added in picogram concentrations to plasma. Furthermore, the ¹²⁵I tracers used in our study were freshly made, ensuring specific binding and optimal sensitivity. Additional explanations could be the variations in the physical condition of the volunteers, duration as well as the degree of exercise between the various studies.

With regard to IL-1, there is more controversy. An elevated baseline IL-1 bioactivity has been reported in highly trained endurance runners (17), and exercise is associated with enhanced IL-1 bioactivity (6). Other

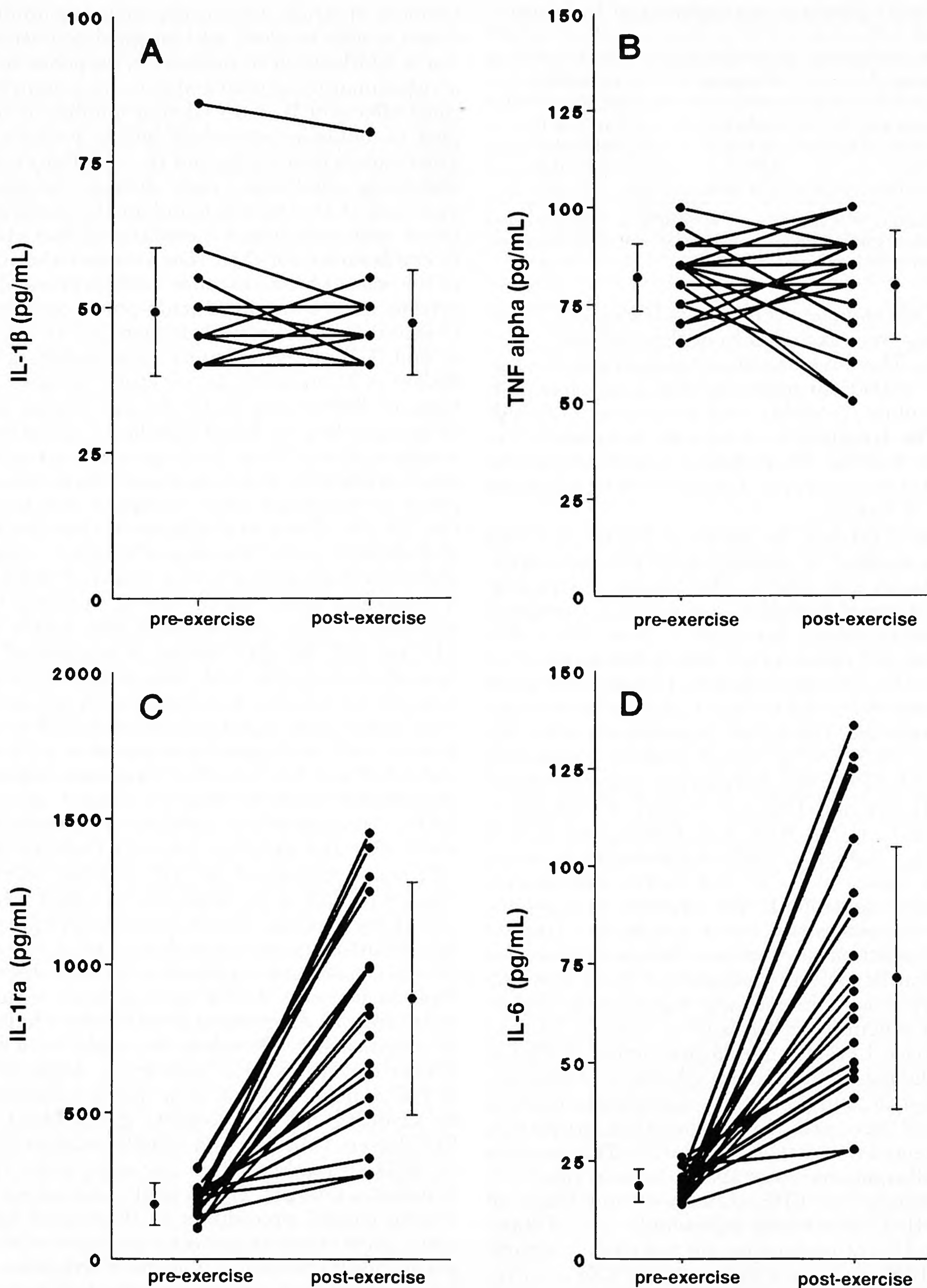


FIG. 1. Circulating interleukin (IL)-1 β (A), tumor necrosis factor (TNF- α ; B), IL-1 receptor antagonist (IL-1ra; C), and IL-6 (D) in 19 athletes. Individual values are represented by black dots and were obtained 24 h before projected end of and immediately after a 6-h endurance run. Bars indicate means \pm SD. Increase was statistically significant for IL-1ra ($P < 0.0005$) and IL-6 ($P < 0.0001$).

studies using ELISA (27, 35) and, like in our study, radioimmunoassays, failed to find an effect of exercise on plasma IL-1 β concentrations. This discrepancy could be due to the fact that the mouse thymocyte

bioassay does not distinguish between IL-1 α , IL-1 β , and IL-6 (41). Indeed, we and others noted elevated IL-6 concentrations in plasma after exercise, which could explain the above-mentioned results (28, 33, 36).

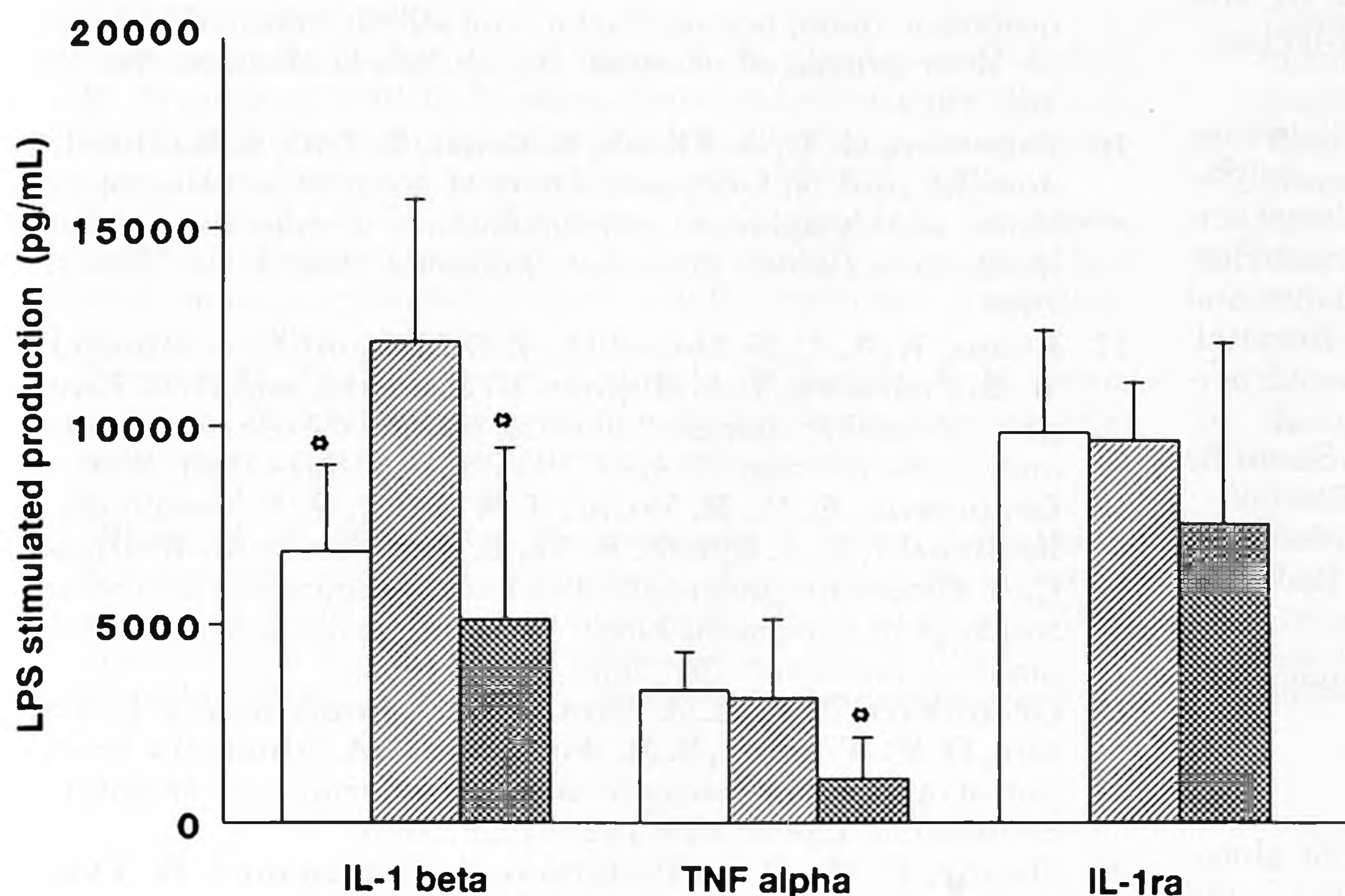


FIG. 2. Production of IL-1 β , TNF- α , and IL-1ra in a whole blood culture system stimulated with lipopolysaccharide (LPS). Data are shown as means \pm SD and are derived from experiments with 19 athletes immediately after (cross-hatched bars) and 24 h before projected end of a 6-h endurance run (hatched bars) and in 10 healthy sedentary controls (open bars). * $P < 0.0005$ compared with preexercise values in athletes.

The LPS-stimulated production of IL-1 β ex vivo at baseline from athletes was significantly higher compared with values obtained in our laboratory from healthy but sedentary controls. This finding cannot be attributed to random temporal changes in cytokine production, since no significant differences were detected in sedentary controls in blood drawn at 2 consecutive days. This suggests that regular training results in an enhanced IL-1 β response to LPS exposure in vitro. We observed that the 6-h endurance run in these well-trained individuals results in a profound decrease of LPS-stimulated IL-1 β and TNF- α production in the whole blood culture system. A similar loss of production capacity of cytokines has been observed in the acute stage of several clinical conditions such as sepsis (27), typhoid fever (24), and after major surgery (25). In studies from our laboratory, this downregulated proinflammatory cytokine response was demonstrated to be associated with a preserved IL-1ra response similar to the one we observed in the current study (24, 25). However, the present results differ from in vitro studies in which isolated mononuclear cells were used (20, 26). In studies of Haahr et al. (20) and Lewicki et al. (26), exercise elicited increased IL-1 β concentrations in supernatants from LPS-stimulated monocytes. (20, 25) In another study, spontaneous release of TNF- α from isolated monocytes was slightly increased at the end of an incremental exercise test but decreased significantly at 20 min after the end of exercise (30). The already mentioned results of Haahr et al. (20) did not find that exercise had an effect on TNF- α production by LPS-stimulated monocytes. Again, there are a number of explanations. As indicated before, differences in the degree and duration of exercise and training background of the volunteers may be crucial. In addition, the whole blood culture system used in our study is somewhat different from culture systems using isolated cells. The whole blood culture system reflects the cytokine response of all types of blood cells to a given stimulus, and this is less artificial and less susceptible

to unwanted ex vivo stimulation than methods using cells isolated by density centrifugation (39).

It is of interest that the downregulation of LPS-stimulated cytokine production is found despite the elevation of number of monocytes, because these cells are considered the major producers of IL-1 β and TNF- α . The downregulation in the whole blood cultures could be due to a plasma factor. In theory, several factors could inhibit cytokine production: cytokines such as transforming growth factor- β (TGF- β), IL-4, and IL-10 have been shown to downregulate TNF- α , IL-1 β , and IL-6 production from LPS-stimulated isolated monocytes (12, 13, 34). Furthermore, TGF- β , IL-4, and IL-10 have been shown to upregulate IL-1ra production (23, 40). Epinephrine and endogenous glucocorticoids are released into the circulation in response to exercise, and both compounds inhibit the release of IL-1, IL-6, and TNF- α from isolated monocytes (1, 25, 30). It is of interest that our ex vivo results are in line with results from in vivo experiments with rats, which show that exercise before exposure to LPS suppresses the TNF response over a period of \sim 6 h (3). Exercise-induced suppression of the LPS-stimulated cytokine response may have both detrimental and beneficial consequences. Curtailing the monocyte production of TNF- α and IL-1 β may compromise the host defense response against invading pathogens on the one hand; on the other hand, it may protect against cytokine-mediated damage of muscle tissue during and after exercise (7, 10). The finding of decreased response to LPS ex vivo is reminiscent of endotoxin tolerance, i.e., the decreased response to a high dose of LPS after pretreatment with a low dose of LPS. It has been found that intense exercise such as performed in a triathlon is associated with a 3.6-fold increase of plasma LPS concentrations in these athletes, perhaps caused by excessive leakage of LPS from the gastrointestinal tract due to reduced splanchnic flow (31). Consequently, it may be suggested that the downregulation of proinflammatory cytokines after exercise is due to endotoxin (18, 19). Further re-

search is needed to elucidate the mechanism of the downregulation of cytokine production after exercise.

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