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Constitutive pro- and anti-inflammatory cytokine and growth factor response to exercise in leukocytes

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[1]*Pediatric Exercise Research Center, Department of Pediatrics, University Children's Hospital, University of California, Irvine;* [2]*Veterans Affairs San Diego Healthcare System, San Diego; Department of Pathology, University of California, San Diego, California;* [3]*University of Tel Aviv, Meir Hospital, Kfar Saba, Israel; and* [4]*Department of Psychiatry, University of California, San Diego, California*

Zaldivar, Frank, Jessica Wang-Rodriguez, Dan Nemet, Christina Schwindt, Pietro Galassetti, Paul J. Mills, Lori D. Wilson, and Dan M. Cooper. Constitutive pro- and anti-inflammatory cytokine and growth factor response to exercise in leukocytes. *J Appl Physiol* 100: 1124–1133, 2006. First published December 15, 2005; doi:10.1152/japplphysiol.00562.2005.—Leukocytosis following exercise is a well-described phenomenon of stress/inflammatory activation in healthy humans. We hypothesized that, despite this increase in circulating inflammatory cells, exercise would paradoxically induce expression of both pro- and anti-inflammatory cytokines and growth factors within these cells. To test this hypothesis, 11 healthy adult men, 18–30 yr old, performed a 30-min bout of heavy cycling exercise; blood sampling was at baseline, end-exercise, and 60 min into recovery. The percentage of leukocytes positive for intracellular cytokines and growth factors and mean fluorescence intensity was obtained by flow cytometry. Proinflammatory cytokines (IL-1α, IL-2, IFN-γ, and TNF-α), a pleiotropic cytokine (IL-6), and anti-inflammatory cytokines and growth factors [IL-4, IL-10, growth hormone (GH), and IGF-I] were examined. Median fluorescence intensity was not affected by exercise; however, we found a number of significant changes ($P < 0.05$ by mixed linear model and modified *t*-test) in the numbers of circulating cells positive for particular mediators. The pattern of expression reflected both pro- and anti-inflammatory functions. In T-helper lymphocytes, TNF-α, but also IL-6, and IL-4 were significantly increased. In monocytes, both IFN-γ and IL-4 increased. B-lymphocytes positive for GH and IGF-I increased significantly. GH-positive granulocytes also significantly increased. Collectively, these observations indicate that exercise primes an array of pro- and anti-inflammatory and growth factor expression within circulating leukocytes, perhaps preparing the organism to effectively respond to a variety of stressors imposed by exercise.

immunology; interleukin-6; growth hormone; intracellular

MANY OF THE HEALTH EFFECTS of exercise are influenced by the balance of stress/inflammatory-related cytokines and growth factors that are known to be altered by physical activity. For example, in elderly humans, higher levels of the circulating cytokine IL-6 are inversely correlated with levels of physical activity, with muscle mass, and with the anabolic mediator IGF-I (3, 19, 54). Brief bouts of heavy exercise also lead to increased numbers of circulating stress/inflammatory cells, such as lymphocytes [T, B, and natural killer (NK) cells], monocytes, and neutrophils, all of which can secrete a variety of cytokines and growth factors (31, 45), and there are new data to suggest that stress activation of circulating leukocytes may play beneficial roles in subsequent poststress challenges, such as tissue injury (55). Leukocytes are increasingly viewed as virtual endocrine, autocrine, or paracrine organs (20), capable of supplying specific tissues with a vast array of mediators that play a role not only in immune responses but also in functions ranging from angiogenesis to bone remodeling (7, 34), all part of the adaptive response to exercise (23, 41). Consequently, the purpose of this study was to examine the impact of brief exercise in healthy young adults on key cytokines and growth factors found within specific leukocyte subpopulations in the circulating blood.

Flow cytometry is used extensively to assess changes in intracellular cytokine production and to identify the specific leukocyte subsets in which these changes are occurring (51). This powerful tool has led to a number of new insights regarding the relationship between intracellular cytokines and leukocyte subtypes. First, it can no longer be assumed that changes in cytokine concentration in the plasma parallel changes observed in those same cytokines within the circulating leukocytes (47). Second, the pattern of intracellular cytokine and mediator response to specific challenges is different within the leukocyte subtypes (e.g., the expression of mediators within monocytes may not parallel the expression of these same mediators in lymphocytes). Thus examining a single population of leukocytes reveals only part of the process.

Most studies involving exercise have examined traditional immune system mediators (24, 27, 35, 44, 46, 48, 49, 52) [e.g., IL-4 and IFN-γ to gauge the impact of exercise on the balance of T-helper-1 (T$_H$1) to T-helper-2 (T$_H$2) type lymphocytes] within a particular leukocyte phenotype (37). Recently, investigators have used flow cytometry to examine lymphocyte expression of heat shock proteins and heme oxygenases in response to exercise (18, 33). Finally, measurement of intracellular IL-6 in monocytes has been used to demonstrate that monocytes were not the source of the increased circulating levels of IL-6 that accompany heavy exercise (47).

There is mounting evidence that the responses to stresses such as exercise likely involve many leukocyte phenotypes and a variety of mediators, even antagonistic mediators. In 1999, Ostrowski et al. (36) noted that strenuous exercise led to increased circulating levels of proinflammatory mediators, but

simultaneously, ". . . cytokine inhibitors and anti-inflammatory cytokines restrict the magnitude and duration of the inflammatory response to exercise." Subsequent investigators have shown that other stressors, such as sepsis (40), also cause a seemingly paradoxical increase in the circulating levels of both pro- and anti-inflammatory cytokines. Studies in adults and children now demonstrate that exercise stimulates growth and stress factors [such as growth hormone (GH) and IL-6] known specifically to antagonize one another (12, 14, 58).

Our laboratory recently demonstrated that a single bout of heavy exercise in young men substantially alters peripheral blood mononuclear cell (PBMC) gene expression characterized in many cases by a brisk upregulation of genes associated with seemingly antagonistic functions of stress, inflammation, and tissue repair (8). Along these lines, hematopoietic cells have been shown to express growth mediators like GH and IGF-I (22, 59) not typically associated with immune function. Accordingly, we hypothesized that brief bouts of heavy exercise would lead to seemingly paradoxical changes in the pattern of intracellular cytokine and growth factor expression in circulating leukocytes, reflecting both pro- and anti-inflammatory function and growth mediators. Based on our studies and studies in other laboratories, we performed multiparameter flow cytometric studies, which examined nine representative cytokines (15, 17) [four proinflammatory/catabolic (IL-1α, IL-2, IFN-γ, and TNF-α), four anti-inflammatory or anabolic (IL-4, IL-10, GH, and IGF-I), and one mixed (IL-6)] in six leukocyte subsets, ranging from neutrophils to monocytes, at three time points (baseline, peak exercise, and recovery).

MATERIALS AND METHODS

Subjects. Healthy young adult men ($n = 11$), between the ages of 18 and 30 yr, were recruited for this study. Exclusion from participation included history of any chronic medical conditions, use of any medications, or training as a competitive athlete. Our Institutional Review Board approved this study, and written, informed consent was obtained from all participants. The studies were conducted at the University of California Irvine General Clinical Research Center (GCRC).

Height, weight and body mass index. Standard, calibrated scales and stadiometers were used to determine height, weight, and body mass index (weight/height^2).

Measurement of cardiorespiratory fitness and exercise intervention. Because many metabolic and immune responses to exercise are "dose" dependent (32), i.e., they are influenced by the relative magnitude and duration of the work input, care was taken to scale the exercise to the capability of each subject. We used an approach that has been successful in normalizing the exercise input in subjects with differing exercise capacities (4). We chose 30 min of heavy exercise [defined as work performed above the subject's anaerobic or lactate threshold (LT)] based on our laboratory's previous experience with healthy nonathletes, because exercising above the LT leads to a robust metabolic and cardiorespiratory response (9).

Subjects reported to the GCRC Applied Physiology-Human Performance Laboratory for two visits on separate days. In *visit 1*, the volunteers performed a ramp-type progressive exercise test on an electronically braked, servo-controlled cycle ergometer to assess maximal exercise tolerance. Gas exchange was measured breath by breath, and the peak oxygen uptake ($\dot{V}O_2$) was determined as previously described using a Vmax-229 system (SensorMedics) (10). The LT was determined according to the method described by Beaver et al. (5). We included those subjects demonstrating a peak $\dot{V}O_2$ in the range of 35–45 ml·min^{-1}·kg^{-1}, a level consistent with a moderate level of fitness. On *visit 2*, separated by at least 48 h, but completed within 1 wk after *visit 1*, subjects were required to exercise for 30 min at a work rate that corresponded to 50% of the difference between the work rate at the LT and peak work rate. This work rate is equivalent to ~80% of the peak $\dot{V}O_2$.

Blood sampling and analysis. During *visit 2*, blood was sampled from an indwelling catheter placed in the antecubital vein. Separate samples were used for complete blood count, ELISA, or flow cytometry assays. The subjects were instructed to sit quietly for 30 min before baseline draw to ensure that measurable physiological parameters of stress (e.g., heart rate and blood pressure) returned to baseline levels after catheter placement. A baseline blood sample was taken before the start of exercise (Pre sampling time point). Subjects then completed the 30-min exercise bout, and a blood sample was obtained immediately following exercise (End sampling time point). The subject was instructed to sit quietly during the recovery phase and was observed by the GCRC nurses. A final blood sample was drawn after 60 min of exercise (Recovery sampling time point). All serum samples used for ELISA assays were aliquoted and stored at −80°C until use. Samples were only thawed once.

Serum lactate, circulating leukocytes, serum cytokines, and growth factors. Serum lactate was measured with the YSI lactate analyzer (YSI 1500, Yellow Springs, OH), with a sensitivity of 0.2 mg/dl. Complete blood counts were obtained by standard methods from the clinical hematology laboratory at our institution.

The following serum factors were measured using commercially available kits manufactured by R&D Systems (Minneapolis, MN) and included the following: IL-1α intra-assay coefficient of variation (CV) 1.1–2.2%, interassay CV 3.4–4.3%, sensitivity ≤1.0 pg/ml; IL-2 intra-assay CV 2.0–4.3%, interassay CV 3.7–5.0%, sensitivity ≤7 pg/ml; IL-4 intra-assay CV 3.4–5.3%, interassay CV 6.1–7.8%, sensitivity ≤10 pg/ml; IFN-γ intra-assay CV 2.6–4.7%, interassay CV 3.7–7.8%, sensitivity ≤8 pg/ml; IL-6 intra-assay CV 3.8–11.1%, interassay CV 9.9–16.5%, sensitivity ≤0.094 pg/ml; IL-10 intra-assay CV 6.6–8.5%, interassay CV 8.1–15.6%, sensitivity ≤0.5 pg/ml; TNF-α intra-assay CV 5.3–8.8%, interassay CV 10.8–16.7%, sensitivity ≤0.12 pg/ml; GH intra-assay CV 3.3–4.3%, interassay CV 6.3–6.5%, sensitivity 0.03 ng/ml; and IGF-I (total circulating IGF-I was detected by extracting IGF-I from IGF-I binding protein by using a modified acid-ethanol extraction method) intra-assay CV 4.5–7.1%, interassay CV 4.8–8.8%, sensitivity 0.03 ng/ml (GH and IGF-I; Diagnostic System Laboratories, Webster, TX). For IL-α, IL-4, and IFN-γ, additional dilutions were made to ensure our ability to measure cytokine levels below the company-provided standard curves.

Antibodies used in flow cytometry to detect surface markers and intracellular mediators. Whole blood was drawn into a vacutainer containing sodium heparin at the Pre, End, and Recovery sampling points. All samples were treated with Golgi stop reagent (Berfeldin A, 10 μg/ml) immediately after phlebotomy and incubated for a total of no more than 4 h before analysis. The samples were not stimulated by mitogens. Surface antigen-specific fluorescent-conjugated monoclonal antibodies (MAbs) CD3 peridinin chlorophyll protein (PerCP)-Cy5.5, CD4 antigen presenting cell (APC), CD8 PerCP-Cy5.5, CD19 APC, and intracellular antibodies to IL-1α phycoerythrin (PE), IL-2 FITC, IL-4 PE, IL-6 FITC, IL-10 PE, IFN-γ PE, TNF-α FITC, and all appropriate isotype controls were purchased from Pharmingen/Becton Dickinson (San Diego, CA). Similar fluorochromes were used in separate tubes to generate data. Anti-GH purchased from ICN Biomedicals (Aurora, OH) and anti-IGF-I purchased from R&D Systems (Minneapolis, MN) were custom conjugated to the appropriate fluorochrome by Bethyl Laboratories (Montgomery, TX).

Staining for cell surface antigens and intracellular cytokines. Briefly, after incubation with Berfeldin A, 100 μl of blood from each sample were added to 12 × 75-mm tubes with specific surface antigen fluorescent-conjugated MAbs, mixed well, and incubated in the dark at room temperature for 15 min. Two milliliters of 1× FACS Lysing Solution (Becton Dickinson, San Jose, CA) were added to lyse red

blood cells, mixed gently, and incubated for 10 min at room temperature in the dark. Two milliliters of 1× wash buffer (Becton Dickinson) were added and centrifuged at 500 *g* for 5 min. Supernatant was removed without disturbing the cell pellet. Five hundred microliters of 1× FACS Permeablizing Solution (Becton Dickinson) were added and mixed well to resuspend the pellet and incubated for 10 min at room temperature in the dark. Three milliliters of 1× wash buffer were added and centrifuged at 500 *g* for 5 min. Supernatants were removed, and fluorescent-conjugated anti-cytokine/growth factor MAbs were added and incubated for 30 min in the dark. Cells were washed as described above and were resuspended in 500 µl of 5% paraformaldehyde.

Acquisition and analysis. Samples were acquired by using a FACS Calibur flow cytometer (Becton Dickinson). All surface and intracellular antibodies were optimized for four-color fluorescent antibody conjugates. CaliBRITE beads and FACSComp software were used for setting the photomultiplier tube voltages and the fluorescence compensation, as well as checking instrument sensitivity before use. A forward scatter threshold was used to acquire 100,000 events for each prepared sample. Flow cytometry data analysis was accomplished by using CellQuest software (version 3.2.1). Specific cell populations (neutrophils, monocytes, and lymphocytes) were delineated by various gating strategies. An example of gating strategy is illustrated in

Fig. 1. Lymphocyte subpopulations were identified by forward and side scatter and separated by antigenic expressions of $CD3^+/CD4^+$ (T-helper lymphocytes), $CD3^+/CD8^+$ (suppressor/cytotoxic T lymphocytes), and $CD19^+$ (B lymphocytes). For intracellular cytokine expressions in NK cells, we gated on the $CD3^-$ and $CD8^+$ dim population. We confirmed that this gated population included $CD3^-$/$CD16^+$ and $CD56^+$ NK cells by surface expression. Monocytes were defined and gated by $CD4^+$ dim vs. side scatter. We found that this gating strategy included 99% of the $CD14^+$ and $CD64^+$ monocytes. The intracellular mediator data were presented in two ways: *1*) in terms of cells positive for a particular mediator (i.e., fluorescence intensity exceeds background) and *2*) as the median fluorescence intensity (MFI).

Statistical analysis. We analyzed the effect of exercise on cytokine and growth mediator regulation in total lymphocytes and their subsets, total monocytes, and total granulocytes in the circulation. We applied a mixed linear model, which is a generalization of the standard linear model and is designed for analyzing repeated measurements. Except for modeling the means, a mixed linear model allows for various patterns of variance among correlated observations, such as data collected repeatedly at different time points from the same subject. In our study, time (Pre, End, Recovery) is our primary explanatory variable, and a quadratic form of time ($time^2$) has been added into the

Fig. 1. An example of the gating strategy used to identify cell populations based on surface $CD4^+$, side scatter complexity (SSC), and intracellular mediators from one subject. *A*: differentiating cell populations based on $CD4^+$ (*Y*-axis markers) and SSC (*X*-axis) of cells using flow cytometry. Monoclonal antibodies in CD and cell side scatter are used to identify distinct cell populations. Cell surface events are expressed in the logarithmic scale on the *Y*-axis from dim expression ($\leq 10^1$) to bright expression ($\geq 10^1$) and scatter on the *X*-axis. 100,000 events are captured for analysis at Pre (baseline blood sample taken before start of exercise), End (blood sample obtained immediately following exercise), and Recovery (blood sample drawn after 60 min of exercise). Four cell populations are clearly identified: L, T-helper cells ($CD4^+$ bright, low side scatter); M, monocytes ($CD4^+$ dim, moderate side scatter); G, granulocytes ($CD4^-$, high side scatter); and N, non-CD4 positive lymphocytes ($CD4^-$, low side scatter). *B*: the $CD4^+$ lymphocyte gate was drawn based on $CD4^+$ bright and low side scatter. *C*: the isotype control labeled with IgG1 phycoerythrin (PE) and IgG2a FITC. Based on the isotype control, the quadrant markers were set for the three samples. *D*: Pre. *E*: End. *F*: Recovery. The percentage of cells positive for growth hormone (GH)/IGF-I in $CD4^+$ cells and the absolute count in the peripheral blood mononuclear cells are listed in the quadrant windows. The absolute count is obtained by multiplying the proportion of $CD4^+$ lymphocytes (%GH or $IGF^+ \times \%CD4^+$) × white blood cell count/µl. APC, antigen presenting cell.
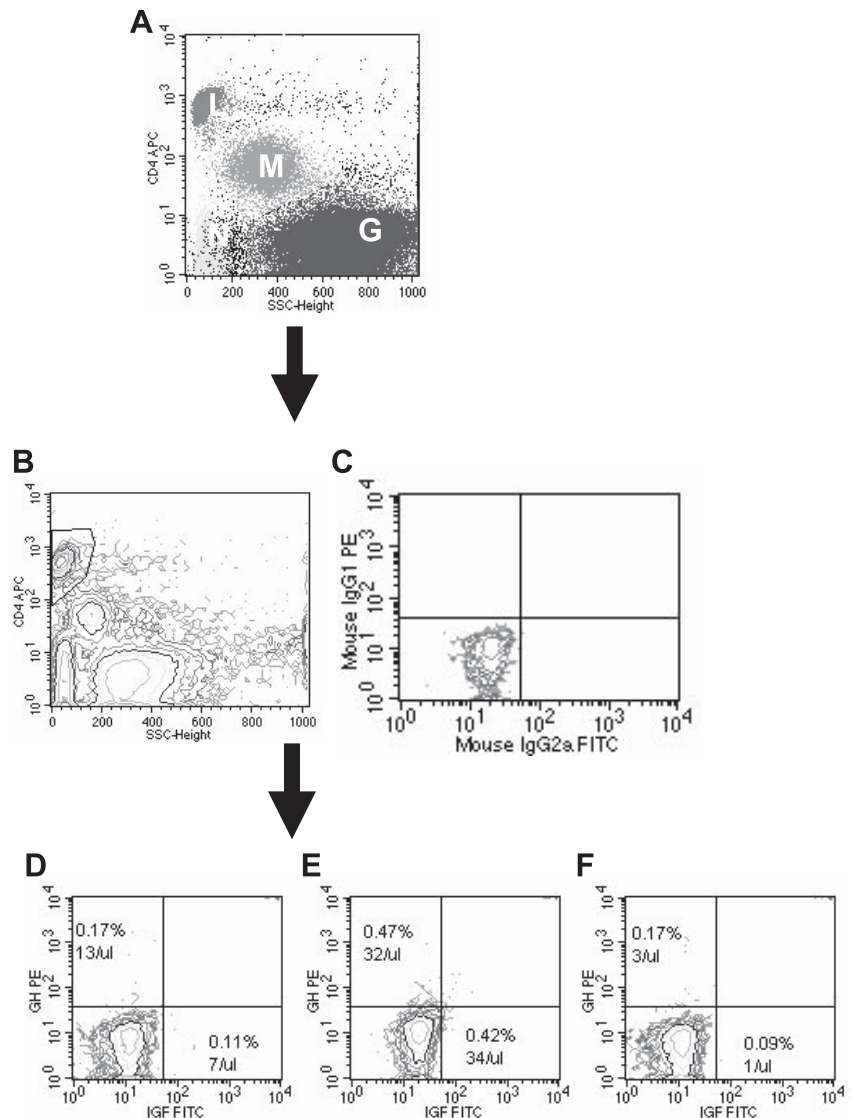
Table 1. *Anthropometric characteristics*

Age, yr	24.8±0.9
Height, cm	176±2.0
Weight, kg	77.2±3.5
BMI, kg/m^2	24.8±1.2
$\dot{V}O_{2peak}$, ml·min^{-1}·kg^{-1}	37.1±1.6
$\dot{V}O_{2peak}$ for constant work rate test, %	80±3.7

Values are means ± SE; $n = 11$ subjects. BMI, body mass index; $\dot{V}O_{2peak}$, peak oxygen uptake.

Table 3. *Impact of exercise on circulating leukocytes*

Circulating Cells	Pre	End-Exercise	Recovery
Total leukocytes	5,809±516*	10,636±1,050‡	6,309±495
Total lymphocytes	1,923±144*	4,726±593‡	1,305±85
Total monocytes	369±53*	674±116‡	316±51
Total granuloctyes	3,412±392*†	5,126±574	4,599±479

Values are means ± SE in cells/μl. Significance at $P < 0.017$: *Pre vs. End; †Pre vs. Recovery; ‡End vs. Recovery.

model for flexibility in determining the pattern of changes over time. For each variable of interest, an overall test of any mean difference between the three time points was first conducted at significance level 0.05; thus we were able to identify a list of variables that showed significant changes in response to exercise. Then, for those significant variables, pairwise comparisons among the three time points (i.e., Pre-End, Pre-Recovery, End-Recovery) were performed and were considered significant if $P = 0.017$ (e.g., 0.05/3). Because little is known about the variability in the response of intracellular cytokines to exercise, we had no data on which to accurately calculate statistical power. Nonetheless, since we found significant changes in response to exercise, these data can be used for future studies to better estimate sample size.

RESULTS

Subjects. Table 1 summarizes anthropometric and exercise test results for the 11 subjects.

Hct and serum lactate. Serum lactate was measured to determine the relative metabolic effect of the external work performed by each subject. The exercise bout caused a 10-fold increase ($P < 0.05$) in serum lactate levels (Pre: 12 ± 0.70 mg/dl vs. End: 121 ± 8 mg/dl, $P < 0.001$) and approached baseline at Recovery (23 ± 2 mg/dl, $P < 0.001$). Mean values for Hct were significantly different at Pre vs. End and End vs. Recovery (Pre: 43 ± 0.8 and 47 ± 0.9, $P < 0.007$; Recovery: 41 ± 0.8, $P < 0.01$).

Serum cytokines and growth factors. We found significant exercise effects on circulating levels of serum cytokines and growth factors (Table 2).

Leukocyte response to exercise. Table 3 summarizes the impact of exercise on total leukocytes, lymphocytes, monocytes, and granulocytes. Table 4 shows the effect of exercise on intracellular levels of the nine inflammatory cytokines and

Table 2. *Effect of exercise on circulating cytokines and growth factors*

Cytokine or Growth Factor	Pre	End-Exercise	Recovery
IL-1α, pg/ml	0.6±0.2*	1±0.2	1±0.2
IL-2, pg/ml	7±2	10±2	9±3
IL-4, pg/ml	1±0.3*	0.8±0.3	0.9±0.2
IL-6, pg/ml	1±0.3*†	3±0.3	4±0.6
IL-10, pg/ml	1±0.4†	2±0.4	3±0.5
TNF-α, pg/ml	2±0.2*	3±0.2‡	2±0.2
IFN-γ, pg/ml	0.6±0.3	1±0.4	1±0.5
IGF-I, ng/ml	269±61	303±54	245±38
GH, ng/ml	0.4±0.3*	23±3‡	2±2

Values are means ± SE. GH, growth hormone; Pre, baseline blood sample taken before start of exercise; End, blood sample obtained immediately following exercise; Recovery, blood sample drawn after 60 min of exercise. Significance at $P < 0.017$: *Pre vs. End; †Pre vs. Recovery; ‡End vs. Recovery.

growth factors within the major groups of leukocytes (i.e., lymphocytes, monocytes, and granulocytes). Table 5 shows the effect of exercise on intracellular levels of cytokines and growth factors within lymphocyte subpopulations. For a variety of reasons, including the cost of performing all of the analyses, we were not able to measure all mediators, as indicated in Table 5 by ND.

Figures 2–5 show the impact of exercise on the proportional changes as a percent change relative to baseline of the key leukocyte cell types and of the specific intracellular cytokines and growth factors. For comparison, the exercise effect on the leukocyte cell type is shown by the thick gray lines, and the changes in individual cytokines and growth factors by the thinner dotted or dashed lines in each of these figures. Finally, we calculated the ratio of T_H2 (T-helper lymphocytes positive for IL-4) to T_H1 (T-helper lymphocytes positive for IFN-γ). There was no significant effect of exercise on this ratio (Pre, 1.1 ± 0.2; End, 1.8 ± 0.6; Recovery, 1.9 ± 0.5).

In contrast to the percent positive cells, we found no significant differences in MFI between any intracellular mediators at Pre, End, and Recovery time points (Tables 6 and 7).

DISCUSSION

By the end of 30 min of heavy exercise in this sample of young adults, the number of stress/inflammatory cells, including T and B lymphocytes, NK cells, monocytes, and neutrophils, had, as expected, increased in the circulating blood. But rather than reflecting a predominantly proinflammatory pattern, the increases in the numbers of cells positive for intracellular cytokines and growth factors within these cells revealed a mixed response in which significant increases in intracellular proinflammatory cytokines (IL-1α, IL-2, TNF-α, and IFN-γ) in at least one of the leukocyte subpopulations were balanced by increases in intracellular anti-inflammatory and anabolic factors (like IL-4, IL-10, GH, and IGF-I). IL-6, a cytokine known to possess both pro- [catabolic (21, 25)] and anti-inflammatory (39) functions and produced directly by exercising skeletal muscle, was significantly changed in monocytes and in all of the lymphocyte subpopulations studied. Thus our data show that the response of leukocytes and their intracellular cytokines and growth factors to exercise in healthy individuals support Ostrowski's hypothesis that the proinflammatory responses to exercise are attenuated by concomitant anti-inflammatory responses.

In the present study, we did not stimulate leukocytes in vitro with immunogenic agents such as lipopolysaccharide. The latter technique is common (38) and is predicated on the notion that immunological function of leukocytes is best observed by measuring their response to a specific antigen or other immunological stimulus. But the goal of this study was to determine

Table 4. *Impact of exercise on intracellular cytokines and mediators in leukocytes*

| Leukocyte Phenotype | Sample | Mediator | | | | | | | | |
| | | Proinflammatory | | | | Mixed | Anti-inflammatory/Anabolic | | | |
		IL-1α	IL-2	TNF-α	IFN-γ	IL-6	IL-4	IL-10	GH	IGF-I
Lymphocytes	P	14±2	14±5*	12±3*	12±2	14±4	17±5	9±2*	41±10	9±2
	E	27±8‡	65±20‡	25±6‡	28±11	20±4‡	27±7‡	42±16‡	55±18	11±4
	R	8±2	15±4	8±2	10±2	10±2	8±2	7±2	26±6	15±6
Granulocytes	P	14±4	12±3	13±3	9±5	13±3	9±2	10±4	3,163±55*†	253±113
	E	14±5	10±4	21±5	11±4	18±3	20±6	9±5	4,743±497	100±34
	R	9±3	19±6	12±5	7±2	16±3	10±3	13±62	4,342±446	199±41
Monocytes	P	7±1†	21±7	6±1	5±1*	8±2	4±1*	18±5	72±40	12±6
	E	6±1	46±18‡	9±2‡	9±2‡	11±2‡	9±1‡	34±10	55±15	6±2
	R	3±1	13±5	3±1	3±1	5±1	2±0.5	19±7	25±9	6±3

Values are means ± SE in positive cells/μl. P, Pre; E, End-exercise; R, Recovery. Significance at $P < 0.017$: *Pre vs. End; †Pre vs. Recovery; ‡End vs. Recovery.

the extent to which exercise itself acted as a stimulus to intracellular cytokine levels in leukocytes, and we, therefore, elected to use flow cytometry to detect intracellular cytokines before, immediately after, and during recovery in unstimulated cells. Other researchers are using this approach as well; for example, Vredevoe et al. (56) recently examined leukocyte expression of various cytokines in patients with heart failure. Like us, those investigators used unstimulated leukocytes and found that the constitutive expression of IL-6 was substantially increased in the patients.

A weakness of the study is the lack of a nonexercised control group. However, we have examined changes in peripheral leukocyte counts over a 30-min rest period for two subjects and observed virtually no changes. There are spontaneous changes in leukocyte counts, but these occur in a circadian pattern over hours and are much smaller in magnitude than the acute changes found with exercise (1). Although our data do not reveal the mechanism for the changes in circulating leukocyte cell types and their intracellular cytokines, some inferences about these mechanisms can be made. As shown in Fig. 5, the proportional increase of granulocytes within the circulation following exercise precisely paralleled the increases in GH-positive granulocytes. A similar parallel proportion was ob-

served for T-helper lymphocytes (Fig. 2A) and their intracellular IL-1α; cytotoxic T-cells (Fig. 3A) and their intracellular IL-6; NK cells (Fig. 3B) and their intracellular IL-6; and monocytes and their intracellular IFN-γ (Fig. 4A). In these cases, our data suggest that exercise mobilized specific cells from pools where these cells were already expressing certain cytokines and growth factors, and the changes in the circulating pool of the leukocyte cell type and their intracellular mediators reflected this proportional mobilization.

Our results also indicate possibly different patterns of mobilization for other leukocyte types and their intracellular cytokines. For example, in a unique case, NK cells and NK cells expressing IL-6 increased substantially following exercise (by ~80–100%), whereas NK cells expressing IGF-I actually fell significantly and remained below baseline by 60 min into recovery (Fig. 3B). In B-cells (Fig. 3C), the proportion of cells expressing GH and IGF-I far exceeded the increases in circulating B-cells alone; similarly, in T-helper cells (Fig. 2), the proportion of cells expressing IL-4, IL-6, IFN-γ, and TNF-α exceeded the increase in T-helper cells. In these cases, leukocytes may have been mobilized disproportionately from pools where the expression of cytokines and growth factors differed.

Table 5. *Impact of exercise on intracellular cytokines and mediators in lymphocytes*

| Lymphocyte Subtype | Sample | Mediator | | | | | | | | |
| | | Proinflammatory | | | | Mixed | Anti-inflammatory/Anabolic | | | |
		IL-1α	IL-2	TNF-α	IFN-γ	IL-6	IL-4	IL-10	GH	IGF-I
CD3+	P	26±4	15±3	ND	ND	17±3	ND	15±3	46±9	22±5
	E	31±7†	40±11	ND	ND	29±4†	ND	24±7	109±48	46±20
	R	11±2	24±9	ND	ND	15±4	ND	13±4	29±8	13±3
CD3+/CD4+	P	21±3	ND	15±5*	16±3	20±4*	16±4*	ND	42±8	20±7
	E	35±8†	ND	46±14†	92±59	66±20†	55±18†	ND	108±40†	31±14
	R	11±3	ND	12±3	8±1	17±4	13±3	ND	24±7	4±1
CD3+/CD8+	P	ND	ND	27±9	22±6	24±6*	22±7	ND	39±8	27±8
	E	ND	ND	51±16	86±50	71±17†	46±17	ND	73±21†	36±15
	R	ND	ND	17±8	10±2	15±3	16±5	ND	25±7	18±8
CD19+	P	ND	ND	ND	ND	ND	ND	ND	11±2*	7±1*
	E	ND	ND	ND	ND	ND	ND	ND	24±6†	31±9†
	R	ND	ND	ND	ND	ND	ND	ND	10±2	8±2
CD8 dim (natural killer cells)	P	ND	ND	56±15	32±8	58±14	27±11	ND	ND	29±8*
	E	ND	ND	61±18	65±36	105±32†	32±15	ND	ND	16±5
	R	ND	ND	19±6	14±4	33±7	11±3	ND	ND	20±8

Values are means ± SE in positive cells/μl. ND, not determined. Significance at $P < 0.017$: *Pre vs. End; †End vs. Recovery.
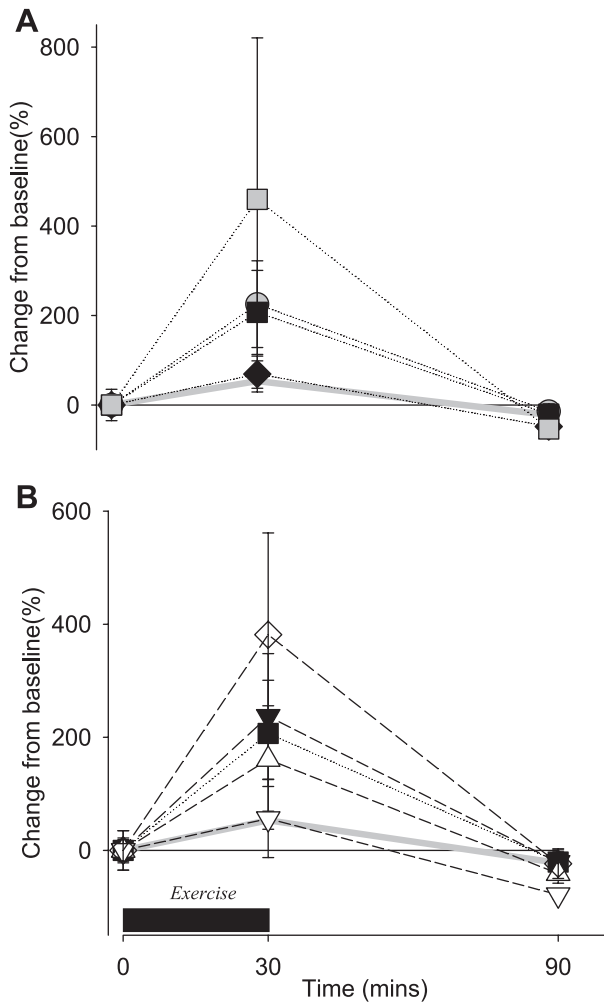
Fig. 2. Effects of exercise on intracellular mediator levels in T-helper lymphocytes (solid thick line). *A*: T-helper-1 proinflammatory: IL-6 (shaded circle), IL-1α (solid diamond), TNF-α (solid square), and IFN-γ (shaded square). *B*: T-helper-2 anti-inflammatory: IL-4 (solid inverted triangle), GH (open triangle), IGF-I (open inverted triangle), and IL-10 (open diamond). Values are means ± SE.

healthy individuals, a balance of T_H1 and T_H2 activity is maintained, despite the overall perturbation in stress/inflammatory cells and their intracellular cytokines. An imbalance between T_H1 and T_H2 cells has been linked to a variety of
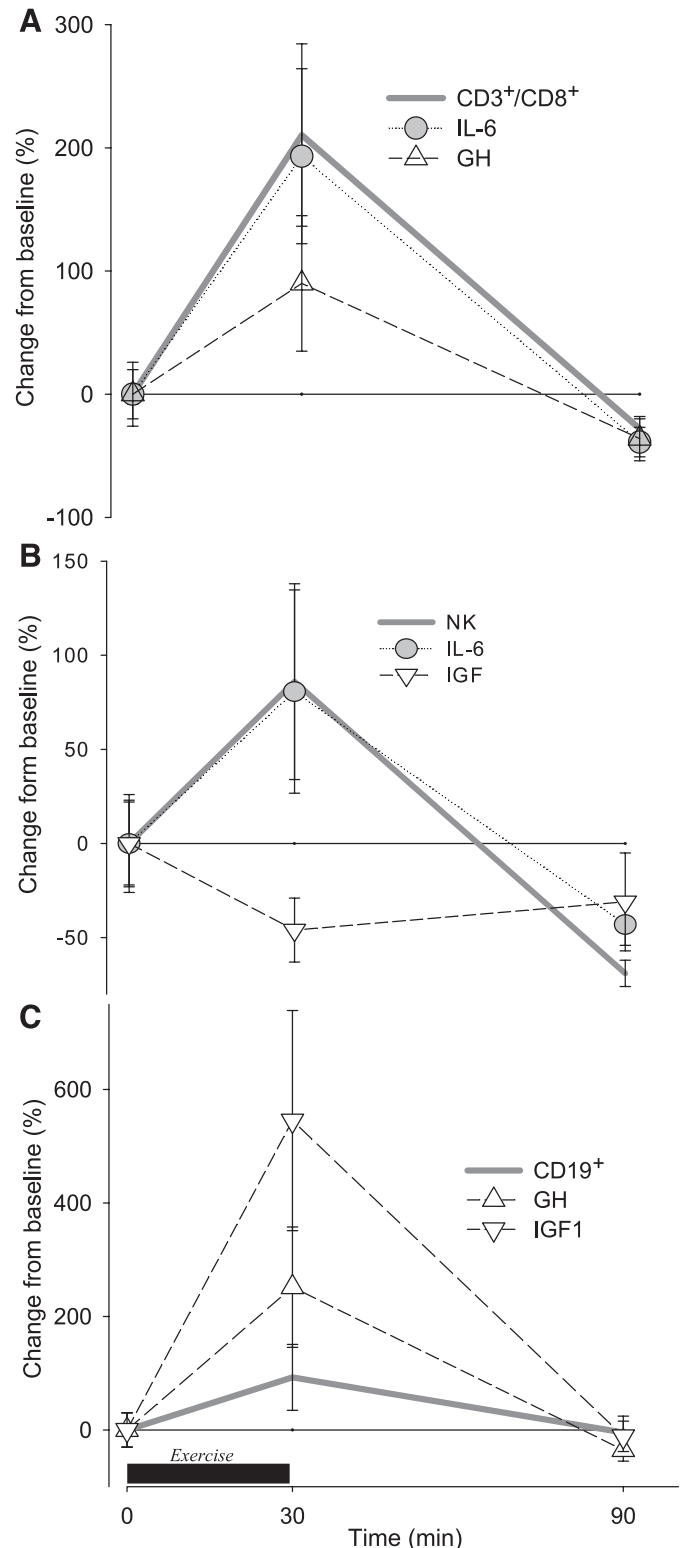

Fig. 3. Effects of exercise on intracellular mediator levels in CD8+ cytotoxic lymphocytes (*A*), natural killer (NK) cells (*B*), and CD19+ B-lymphocytes (*C*). Values are means ± SE.

Alternatively, exercise itself (perhaps through the effect of circulating factors like GH or IL-6) influences the intracellular expression of cytokines. In contrast to the number and proportion of cells positive for a given mediator, we found no effect of brief exercise on MFI (Tables 6 and 7). This would tend to support the idea that the predominant influence of exercise is to mobilize distinct populations of cells from marginal pools where they are already expressing mediators at a particular level, rather than altering the cytokine production of the cells themselves. The mechanism of this remarkable regulation of the mobilization of leukocytes and their intracellular cytokines has yet to be elucidated.

Our data suggest that the leukocyte-associated proinflammatory and anti-inflammatory response to exercise is robust, multifactorial, and brief, all features consistent with the global stress/immune activation associated with the "danger" concept of initial immune regulation, leading, ultimately, to either tolerance or activation (28). We found, for example, that T_H2 cells increased significantly with exercise, but the ratio of T_H1 to T_H2 cells did not significantly change, suggesting that, in
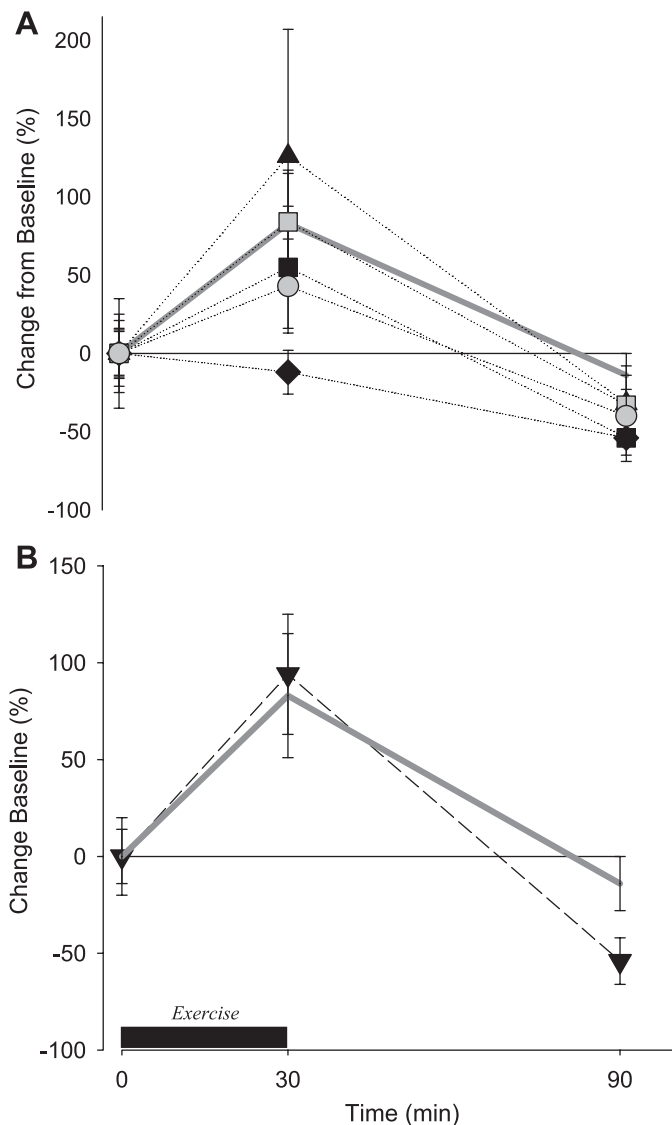
Fig. 4. Effects of exercise on intracellular mediator expression in monocytes (solid thick line). *A*: proinflammatory: IL-1α (solid diamond), IL-2 (solid triangle), TNF-α (solid square), IFN-γ (shaded square), and IL-6 (shaded circle). *B*: anti-inflammatory: IL-4 (solid inverted triangle). Values are means ± SE.

suggests that these growth factors can aid in angiogenesis, tendon strength, tissue healing, and repair, all functions that are important in the adaptation to exercise (13, 16, 29). In addition, GH serves as a cytokine by aiding in lymphoid proliferation and preventing stress-induced apoptosis (26).

White cells are known to migrate to exercising tissue during the recovery phase (42). A recent study by Viswanathan and Dhabhar (55) elegantly demonstrated that migration of leukocytes to the site of a surgical wound is significantly increased in those animals that had been psychologically stressed. Whether or not intracellular GH or IGF-I act in an autocrine or paracrine manner to promote tissue adaptation to exercise has not been studied. Of related interest is the role of circulating angiogenic precursor cells that are now known to be stimulated by exercise (43) and capable of supplying key growth factors to support new capillary formation in a localized manner (2).

Our data demonstrate that exercise-induced changes in the intracellular leukocyte cytokines do not necessarily parallel the changes in circulating levels of those same cytokines and growth factors. The largest increases in circulating cytokines and growth factors occurred for GH and IL-6 (Table 2), which are known to be produced in response to exercise by the pituitary and skeletal muscle, respectively. No significant increase was found for circulating IGF-I, IL-2, or IFN-γ, despite relatively large and significant changes in intracellular expression of these mediators in some cell types. We did find significant increases in circulating levels of the proinflammatory cytokine TNF-α and the anti-inflammatory cytokine IL-10, and a significant decrease in IL-4. These observations also support Ostrowski's notion that the initial stress/inflammatory response to exercise involves both pro- and anti-inflammatory activity. Of the cytokines we examined, IL-6 is the mediator most consistently reported to be elevated within the circulation after exercise. In the case of the other mediators that we measured, circulating levels seem to be more sensitive to a variety of factors, including exercise intensity, type, and dura-

diseases, including allergy and asthma (30). An intriguing, but as yet unanswered, question is whether or not individuals who are susceptible to the common entity of exercise-induced asthma (11) maintain T_H1/T_H2 balance in response to brief exercise as did our nonasthmatic subjects (note: none of our subjects experienced wheezing following the exercise test).

GH and IGF-I have been detected in stress and immune cells (22, 59), but no previous studies have examined the impact of brief exercise on the numbers of GH or IGF-I-positive cells in the circulation. Granulocytes positive for GH increased significantly with exercise, as did GH-positive B-cells. IGF-I expressing B-cells also increased, but, as noted above, IGF-I-positive NK cells decreased following exercise, despite an overall increase in the numbers of circulating NK cells themselves. The importance of GH and IGF-I in the maintenance of muscle mass is well documented (53); more recent work also
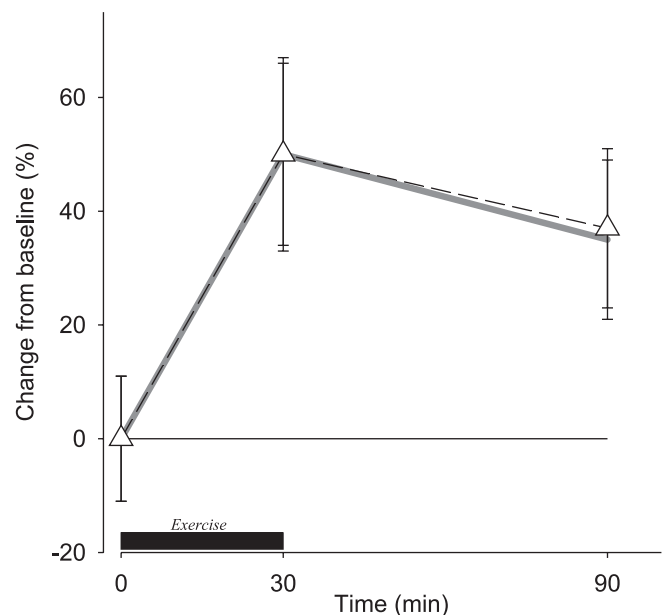

Fig. 5. Effects of exercise on intracellular mediator expression in granulocytes (solid thick line). GH (open triangle) is shown.

Table 6. *Impact of exercise on intracellular cytokines and mediators in lymphocytes*

| | | Mediator | | | | | | | | |
| | | Proinflammatory | | | | Mixed | Anti-inflammatory/Anabolic | | | |
Lymphocyte Subtype	Sample	IL-1α	IL-2	TNF-α	IFN-γ	IL-6	IL-4	IL-10	GH	IGF-I
CD3$^+$	P	8±1	10±1	ND	ND	3±0.3	ND	9±3	23±8	31±5
	E	7±1	14±3	ND	ND	4±0.4	ND	10±4	14±6	32±5
	R	7±1	15±3	ND	ND	5±1	ND	14±5	18±6	29±6
CD3$^+$/CD4$^+$	P	7±1	ND	5±1	16±3	9±5	9±1	ND	9±1	28±6
	E	7±1	ND	8±3	15±3	14±7	9±2	ND	8±1	25±6
	R	11±2	ND	20±6	14±2	9±5	10±2	ND	7±1	12±5
CD3$^+$/CD8$^+$	P	ND	ND	5±1	19±3	9±6	10±1	ND	10±1	35±7
	E	ND	ND	5±1	21±3	10±6	10±1	ND	7±1	36±7
	R	ND	ND	9±4	20±4	12±2	11±1	ND	14±5	33±6
CD19$^+$	P	ND	ND	ND	ND	ND	ND	ND	48±13	45±10
	E	ND	ND	ND	ND	ND	ND	ND	20±4	29±7
	R	ND	ND	ND	ND	ND	ND	ND	31±13	33±11
CD8 dim (natural killer cells)	P	ND	ND	5±1	22±4	10±6	11±2	ND	ND	37±7
	E	ND	ND	9±3	21±4	10±4	11±4	ND	ND	34±8
	R	ND	ND	16±6	23±5	21±8	13±2	ND	ND	32±7

Values are means ± SE in median fluorescence intensity.

tion; changes in plasma volume (note: we did find, as expected, an increase in Hct following exercise in our subjects); and, perhaps, the sensitivity of the assay used for detection (6). The extent to which mediators originating from circulating leukocytes contribute to circulating levels of these mediators is simply not known.

Our data show a small change in Hct with the relatively heavy exercise bout. Some investigators have used these changes as a way of estimating changes in plasma volume and as evidence of hemoconcentration (57). In humans, as in other mammals, heavy exercise is accompanied by an infusion of highly concentrated red blood cells from the spleen, and the use of Hct to "correct" for concentrations of other mediators in the blood has been recently questioned (50). The observed changes in Hct probably have little if any substantial impact on our data. For example, in the case of circulating cytokines, IL-4 significantly decreased, the opposite of the effect of hemoconcentration. Moreover, the mean change in Hct was only 8.7%, whereas the percent increases in cytokines were 40% for IL-1α, 228% for IL-6, 64% for IL-10, and 580% for GH. Similarly, the percent changes for intracellular cytokines ranged roughly from 25 to 600%.

In summary, the current data show that the constitutive response of intracellular mediators within stress/inflammatory cells, traditionally thought to be part of a proinflammatory response, actually represent a balanced mixture of pro- and anti-inflammatory mediators, consistent with the paradoxical nature of the immediate immune response to brief exercise. These results corroborate our recent findings that a single bout of heavy exercise (in a separate group of young adults using the same protocol as outlined here) substantially altered expression of over 300 genes in PBMCs, characterized by a brisk activation and deactivation of genes associated with stress, inflammation, and tissue repair (8). Even with a focus on only nine cytokines and growth factors, we found that brief exercise led to substantial changes in the numbers and intracellular cytokine expression of leukocytes in the circulating blood. Cells positive for the predominantly proinflammatory cytokines IL-1α, IL-2, IFN-γ, and TNF-α were increased, but so were cells positive for anti-inflammatory and growth mediators like IL-4, IL-10, GH, and IGF-I. It thus appears that brief, heavy exercise leads to a sizeable mobilization of leukocytes expressing a variety of mediators, some of which appear to be antagonistic.

Collectively, these observations indicate that exercise increases the numbers of circulating cells positive for both pro/anti-inflammatory and growth factors, perhaps contributing to the organism's ability to effectively respond to a variety of postexercise challenges, such as tissue injury and adaptation.

Table 7. *Impact of exercise on intracellular cytokines and mediators in leukocytes*

| | | Mediator | | | | | | | | |
| | | Proinflammatory | | | | Mixed | Anti-inflammatory/Anabolic | | | |
Leukocyte Phenotype	Sample	IL-1α	IL-2	TNF-α	IFN-γ	IL-6	IL-4	IL-10	GH	IGF-I
Granulocytes	P	32±5	33±4	32±19	126±32	12±2	71±24	41±8	133±29	74±11
	E	30±5	56±8	13±1	137±33	12±2	51±12	40±8	159±27	84±16
	R	28±5	48±11	18±6	127±25	9±1	75±24	47±11	120±24	92±18
CD4 dim (monocytes)	P	19±3	23±2	8±1	40±7	8±1	18±3	22±7	28±3	12±6
	E	16±1	23±2	17±8	41±8	8±1	31±10	14±1	23±4	6±2
	R	17±5	22±3	17±8	34±8	9±1	29±10	15±1	37±8	6±3

Values are means ± SE in median fluorescence intensity.

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