

Endurance exercise causes interaction among stress hormones, cytokines, neutrophil dynamics, and muscle damage

KATSUHIKO SUZUKI,¹ MANABU TOTSUKA,² SHIGEYUKI NAKAJI,¹
MUTSUO YAMADA,¹ SATORU KUDOH,¹ QIANG LIU,¹
KAZUO SUGAWARA,¹ KANEMITSU YAMAYA,³ AND KOKI SATO²

¹Department of Hygiene, Hirosaki University School of Medicine, Hirosaki, Aomori 036-8562; ²Department of Health and Physical Education, Faculty of Education, Hirosaki University, Hirosaki, Aomori 036-8560; and ³Oyokyo Kidney Research Institute, Hirosaki, Aomori 036-8243, Japan

Suzuki, Katsuhiko, Manabu Totsuka, Shigeyuki Nakaji, Mutsuo Yamada, Satoru Kudoh, Qiang Liu, Kazuo Sugawara, Kanemitsu Yamaya, and Koki Sato. Endurance exercise causes interaction among stress hormones, cytokines, neutrophil dynamics, and muscle damage. *J. Appl. Physiol.* 87(4): 1360–1367, 1999.—We analyzed adaptation mechanisms regulating systemic inflammatory response of the stressed body by using an experimental challenge of repeated exercise bouts and accompanying muscle inflammation. Eight untrained men bicycled at 90 W for 90 min, 3 days in a row. Exercise induced peripheral neutrophilia with a leftward shift of neutrophil nucleus and neutrophil priming for oxidative activity determined by luminol-dependent chemiluminescence. Plasma growth hormone and interleukin-6 rose significantly after exercise and were closely correlated with the neutrophil responses. Serum creatine kinase and myoglobin levels as muscle damage markers rose after exercise in “delayed onset” and were closely correlated with the preceding neutrophil responses. These exercise-induced responses were strongest on *day 1*, but the magnitude gradually decreased with progressive daily exercise. In contrast, the magnitude of catecholamine responses to exercise sessions gradually rose, possibly suppressing neutrophil oxidative responses. These results indicate that stress-induced systemic release of bioactive substances may determine neutrophil mobilization and functional status, which then may affect local tissue damage of susceptible organs.

growth hormone; interleukin-6; catecholamine; reactive oxygen species; systemic inflammatory response syndrome

HOMEOSTASIS IS MAINTAINED on exposure to stress through adaptation mechanisms such as nervous and endocrine systems, which Walter Cannon and Hans Selye long ago established as “stress theory” (7, 13). However, pathophysiological mechanisms disturbing homeostasis after acute stress-related diseases, leading to fatal multiple-organ failure, are not yet well understood (1). Systemic inflammatory response syndrome (SIRS) is a new concept, integrating clinically serious diseases such as severe trauma, burn injury, shock, systemic infections, adult respiratory distress syndrome, acute pancreatitis, and myocardial infarction on the basis of systemic cytokine release (hypercytokinemia) (1). Pathogenic effectors of SIRS are considered to be reactive

oxygen species (ROS) and lysosomal enzymes released mainly from circulating neutrophils, because these cells are strongly primed or activated by cytokines (2, 14) and adversely affect microcirculation and destroy tissues (14, 17, 20). Viewing SIRS as an extension of stress theory may prove promising in explaining the mechanisms of homeostasis destruction due to extreme physical stress.

SIRS indicates the presence of two or more of the following: 1) body temperature exceeding 38°C; 2) heart rate exceeding 90 beats/min; 3) respiration exceeding 20 breaths/min; 4) leukocyte count exceeding 12,000 cells/ μ l or less than 4,000 cells/ μ l; and 5) immature cells (band neutrophils) exceeding 10% (leftward shift of neutrophil nucleus) (1). Interestingly, exhaustive endurance exercise such as a marathon (26b) meets all of the above requirements for SIRS. Exercise as such does not result in multiple-organ dysfunction as an ultimate consequence of SIRS, but “delayed onset” muscle soreness and injury are often experienced after unaccustomed exercise, indicating the inflammatory tissue damage (28). Exercise disrupts skeletal muscle ultrastructurally, resulting in neutrophil infiltration and release of myocellular proteins such as creatine kinase (CK) and myoglobin (Mb) into the circulation (9, 26b, 28). Systemically, marked neutrophilia with a leftward shift (11, 24) and enhanced capacity of neutrophils to produce ROS have been documented after endurance exercise at >60% maximal oxygen uptake ($\dot{V}O_{2\max}$) for longer than 60 min (16, 22, 24, 26; see also review in Ref. 25). In addition, researchers have reported that exercise-induced peripheral neutrophilia was correlated positively with, but appeared earlier than, CK efflux (5, 19, 26). This strongly suggests that circulating neutrophils mobilized and primed after stressful exercise may be an important mediator causing muscle damage after exercise, as is the case with SIRS.

As to whether exercise stimulates cytokine release, Cannon et al. (3) first reported that plasma interleukin-1 (IL-1) activity was detected after endurance exercise (60% $\dot{V}O_{2\max}$, 60 min). On the basis of this, Smith et al. (22) proposed that neutrophil priming after exercise identical to that in Cannon et al. (60% $\dot{V}O_{2\max}$, 60 min) might reflect the exercise-induced systemic cytokine release, but they denied the hypothesis later, because they could not detect cytokines in plasma (21). However, delayed-onset secretion of several cytokines has

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

been demonstrated, whether immediately after “prolonged” endurance exercise exceeding 60 min or during the “recovery” period several hours after short-duration intensive or muscle-damaging exercise (3, 5, 6, 8, 18, 26a, 26b), the time course changes of which after exercise resemble the findings of neutrophil oxidative responses as reviewed in a previous study from our laboratory (25). Furthermore, stress hormones such as catecholamine, cortisol, and growth hormone (GH) have also been implicated as not only causative mediators of exercise-induced cytokine secretion (3, 18) but also modulators of neutrophil count and function (13, 24, 25, 26b). Therefore, we hypothesized that communications exist among nervous, endocrine, cytokine, and neutrophil behavior as underlying mechanisms of exercise-induced muscle damage.

Exercise is recommended in stress research because of noninvasive loading, the reproducible and quantifiable experimental workload, as findings that acute, intense exercise triggers more striking elevations in concentrations of circulating immune cells and stress hormones than do other stress models (13). We studied involvement of main-candidate hormones and cytokines that mobilize and/or prime circulating neutrophils in exercise-induced muscle damage by using more prolonged exercise than in the above-mentioned studies, including a several-hours-long “recovery” period. By using such a subclinical situation experimentally on the basis of changes over time, our study of the regulatory mechanisms for SIRS was clear cut compared with clinical studies of complicated, extreme situations. Previous work showed that neutrophil mobilization and priming and CK release after endurance exercise were attenuated by daily repeated exercise at the same “relative” workload, reducing interindividual and day-to-day workload variation (27). In this study, we wanted to clarify the variance for exploring interactions among parameters, using consecutive sessions of endurance exercise loaded at the same “absolute” intensity. Our data confirmed the above hypothesis and substantiated the concept that the endogenously created balance between agonistic and antagonistic substances in response to stress may determine neutrophil mobilization and functional status, which then may affect inflammation of susceptible organs.

METHODS

Subjects and exercise protocol. After approval of experimental procedures by the Ethics Committee of the Hiroshima University School of Medicine, informed consent was obtained from eight healthy male student volunteers with minimal athletic training background [age: 19–21 yr; height: 170.8 ± 3.7 (SD) cm; body mass: 64.4 ± 15.7 kg]. On a day at least 1 wk before the exercise sessions, each subject took a maximal exercise test by using a protocol of incremental graded increase in workload on a cycle ergometer until exhaustion (Takei, Tokyo, Japan) to determine $\dot{V}O_{2\max}$, as previously described (24). From data on $\dot{V}O_{2\max}$ (3.10 ± 0.48 l/min, corresponding to 49.6 ± 9.9 ml·kg⁻¹·min⁻¹), subjects were considered untrained.

Exercise protocol and blood sampling. No subjects conducted strenuous physical activity (sedentary state in day-

time classes) before or during the experiment except for exercise sessions. Subjects completed 90-min bicycling sessions at the identical absolute intensity, with a power output held at 90 W for 3 consecutive days. All exercise bouts were begun between 6:00 and 8:00 PM, when cortisol, one of the most immunosuppressive hormones, is stable in the falling phase of the body's circadian rhythm (7, 27). During exercise, the heart rate was continuously monitored with a PE-3000 Sports Tester (Polar Electro, Kempele, Finland). Oxygen uptake ($\dot{V}O_2$) in subjects on the bicycle was intermittently monitored each 30 min on the basis of the Douglas bag method, as described previously (24). The percentage of workload to the individual $\dot{V}O_{2\max}$ was calculated for each subject by dividing $\dot{V}O_2$ data (ml/kg) by $\dot{V}O_{2\max}$. Peripheral venous blood samples (12 ml) were drawn through an indwelling venae-catheter (Angiocath, Becton Dickinson, Sandy, UT) placed in a forearm vein on *day 1* and by antecubital venipuncture 12 h (the next morning) after exercise completion (Post 12 h) and before (Pre), immediately after (Post), and 1 h (Post 1 h) and 12 h (Post 12 h; the next morning) after exercise on *days 2* and *3*.

Total and differential leukocyte count. Total leukocyte count in EDTA-treated blood was measured by using a Sysmex microcell counter F-300 (TOA Medical Electronics, Kobe, Japan). The coverslipped smears were prepared on freshly drawn whole blood immediately after blood sampling in the absence of anticoagulant and Wright-Giemsa stained as described before (25). The different leukocyte types were classified into band and segmented neutrophils, lymphocytes, monocytes, eosinophils, and basophils by using oil-immersion magnification ($\times 1,000$) from at least 200 cells/slide. The absolute number of each cell type was calculated from the total leukocyte count and the percentage of each differential count.

Luminol-dependent chemiluminescence (CL). Neutrophils were isolated from heparinized peripheral blood by one-step discontinuous density gradient centrifugation by using Histopaque-1077 and -1119 (Sigma Chemical, St. Louis, MO) as described previously (12, 25). Harvested neutrophils were washed once with Hanks' balanced salt solution and resuspended in Hanks' balanced salt solution at 3×10^6 cells/ml. For an in vitro stimulant, opsonized zymosan (OZ) at a concentration of 1 mg/ml and 1×10^{-6} M phorbol 12-myristate 13-acetate (PMA; Sigma Chemical) were used. Luminol was prepared by dissolving 5-amino-2,3-dihydro-1,4-phthalazinedione (Sigma Chemical) to a final concentration of 2 mM. These reagents were prepared as previously described in detail (12, 25) with adequate measurement conditions confirmed for comparing cellular responsiveness (12). Neutrophil suspensions of 0.1 ml were added to polystyrene cuvettes and incubated at 37°C with 0.1 ml of luminol solutions. At *time 0*, 0.4 ml of the cell stimulant was added to the mixture, and the CL response was recorded at 37°C in a Lumiphotometer TD-4000 (Labo Science, Tokyo, Japan) at ~ 2 -min intervals. The peak value in the monophasic response curve (maximum CL intensity) was analyzed for each limb by using the least squares method and expressed as peak height (mV) as described previously (24, 25).

Plasma substance measurement. Plasma samples were separated from whole blood, using EDTA as an anticoagulant immediately after blood sampling, by centrifugation at 1,000 g for 10 min and stored frozen at -80°C . PMN elastase (elastase- α_1 -proteinase-inhibitor complex) as a marker for neutrophil granule content release in plasma was measured with solid-phase enzyme immunoassay (Merck, Darmstadt, Germany). IL-1 β , tumor necrosis factor- α (TNF- α), and interferon- γ (IFN- γ) were measured with enzyme-amplified sensi-

tivity immunoassay (EASIA) kits (Medgenix, Fleurus, Belgium) on the basis of the ELISA principle. IL-6 and IL-8 were determined with ELISA kits (Medgenix). The assay sensitivity limit was IL-1 β (2 pg/ml), IL-6 (3.5 pg/ml), IL-8 (3 pg/ml), TNF- α (3 pg/ml), and IFN- γ (0.1 IU/ml). GH was measured with an immunoradiometric assay kit (Eiken Chemical, Tokyo, Japan). Cortisol was measured with a CL enzyme immunoassay kit (Sanofi Diagnostics Pasteur, Chaska, MN). Epinephrine, norepinephrine, and dopamine were measured by using a radioenzymatic assay (Catecholamine Research Assay System, Amersham, Bucks, UK). Area under the curve (AUC) analysis was used to evaluate time course kinetics of plasma substances in data from Pre to Post 1 h.

Serum biochemistry. Serum samples were separated from whole blood by using Vacutainer blood-collection tubes (Becton Dickinson, Franklin Lakes, NJ) by centrifugation at 1,000 *g* for 10 min after the blood was allowed to clot at room temperature for 30 min. These samples were stored frozen at -80°C until assayed. Serum CK activity was measured by using biochemical assay kits prescribed for the kinetic enzyme analyzer Paramax (Baxter Diagnostics, Deerfield, IL). Mb concentration was measured by using CL enzyme immunoassay kits (Luminomaster Mb, Sankyo, Tokyo, Japan); the sensitivity limit was 20 ng/ml. C3 and C4 were determined by using nephelometry rate kits (Kyowa Medics, Tokyo, Japan).

Statistics. Data are presented as means \pm SD. Statistical validations for time course changes were made by a two-way analysis of variance. To test whether the magnitude of exercise-induced changes was altered depending on days, a two-way analysis of variance with repeated measures was employed. These analyses were followed by Scheffé's adjustment after significant interactions were found. Associations between experimental variables were studied by calculating Pearson's correlation coefficient (*r*). Significance was evaluated in all statistics at $P < 0.05$ or $P < 0.01$.

RESULTS

Physiological data. $\dot{V}O_2$ values during 30- and 60-min exercise and Post were 1.49 ± 0.16 , 1.44 ± 0.15 , and 1.65 ± 0.13 l/min ($P < 0.05$), respectively, indicating that oxygen demand was relatively higher at the last stage of exercise. On the other hand, heart rate values during 30- and 60-min exercise and Post were 147 ± 17 , 146 ± 13 , and 138 ± 14 beats/min, respectively, tending downward. The mean relative intensity of exercise was calculated to give $52.8 \pm 10.8\%$ $\dot{V}O_{2\max}$ on *day 1*, and no significant differences were seen in these parameters among *days 1-3*.

Total and differential leukocyte counts. Leukocyte counts rose significantly from those during 30-min exercise until Post 3 h but returned to the preexercise level at Post 12 h (Fig. 1). Leukocytosis was attributed to neutrophilia, and the band neutrophil-to-total neutrophil ratio as an index of a leftward shift increased significantly at Post 1 h ($18.6 \pm 6.7\%$, $P < 0.01$) compared with the Pre value ($12.6 \pm 4.8\%$). The band neutrophil-to-total leukocyte ratio, as one of the diagnostic criteria of SIRS ($\geq 10\%$), was also prominent at Post 1 h ($13.8 \pm 5.6\%$, $P < 0.01$) and Post 3 h ($11.7 \pm 5.6\%$, $P < 0.01$) compared with the Pre value ($6.9 \pm 3.1\%$). The numbers of lymphocytes, monocytes, eosinophils, and basophils did not change significantly. Leukocytosis due to neutrophilia was marked on *day 1* and somewhat reduced on *days 2* and *3* (Fig. 1), but there

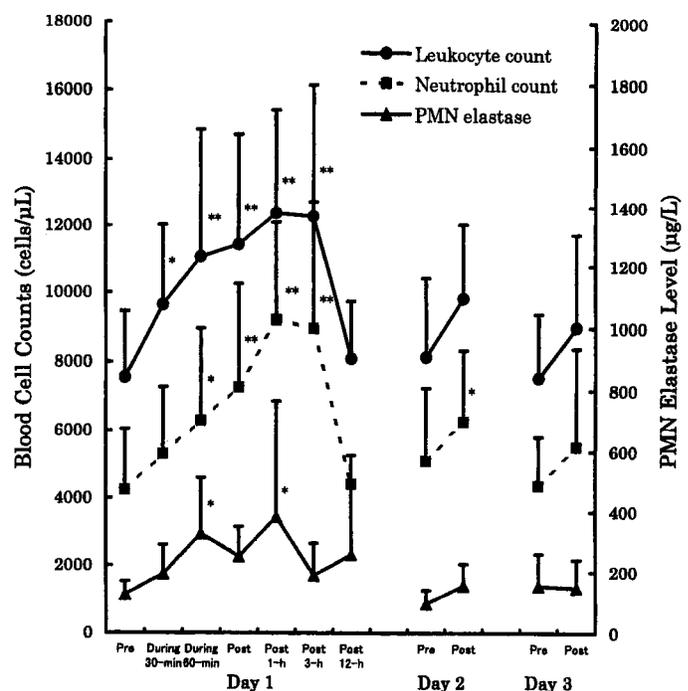


Fig. 1. Effects of 3 consecutive daily sessions of same-intensity endurance bicycle exercise (90 W, 90 min) on circulating leukocyte count, neutrophil count, and plasma polymorphonuclear neutrophil (PMN) elastase concentration in untrained men. Values are means \pm SD. Pre, at rest before exercise; Post, Post 1 h, Post 3 h, and Post 12 h: immediately after and 1, 3, and 12 h after exercise, respectively; During 30- and 60-min, during 30- and 60-min exercise, respectively. *Significantly different from each Pre value, $P < 0.05$. **Significantly different from each Pre value, $P < 0.01$.

were no significant differences in response magnitude among days.

PMN elastase. Plasma PMN elastase rose during 60-min exercise and Post 1 h ($P < 0.05$) on *day 1*, but the increment tended to disappear with daily repetition (Fig. 1). The changing pattern almost corresponded to the neutrophilia as the source of release. When PMN elastase data were divided by the neutrophil count, exercise-induced significant enhancement disappeared (data not shown), indicating that the PMN elastase release on a per-cell basis was not stimulated after exercise.

Neutrophil oxidative activity. The capacity of neutrophils to produce ROS was measured by use of a fixed number of cells isolated from peripheral blood. OZ-stimulated CL response was significantly enhanced from that during 60-min exercise and continued at least until Post 3 h ($P < 0.01$) (Fig. 2). PMA-stimulated CL response was relatively stable but rose significantly at Post and Post 3 h ($P < 0.01$) on *day 1* (Fig. 2). These increments in response after exercise were not noted on *day 3*, especially in PMA-stimulated CL response (*day 1* vs. *day 3*, $P < 0.01$) (Fig. 2).

Cytokines. The plasma concentrations of IL-1 β , TNF- α , and IFN- γ were stable and did not change significantly after exercise sessions (Table 1). With wide interindividual variations, the IL-6 level increased in a biphasic pattern at Post ($P < 0.05$), increased more markedly at Post 3 h and Post 12 h ($P <$

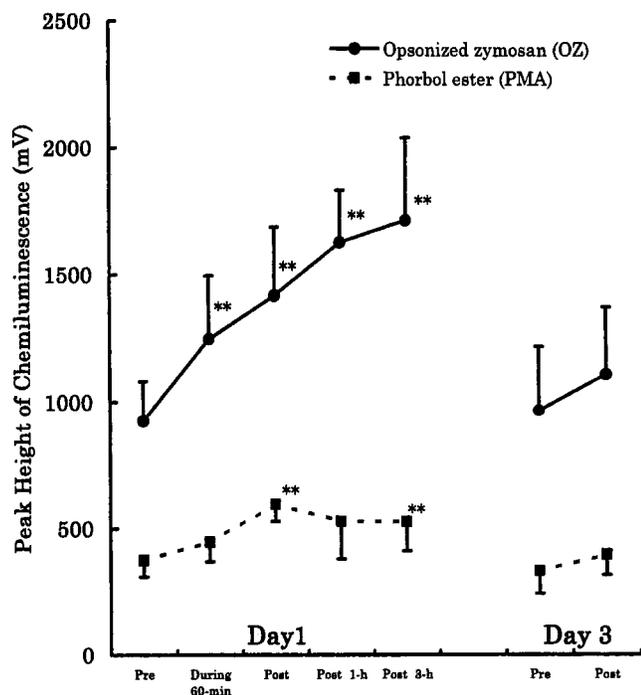


Fig. 2. Endurance exercise-induced changes in capacity of isolated neutrophils (3×10^5 cells) to produce reactive oxygen species assessed by luminol-dependent chemiluminescence on stimulation with opsonized zymosan (OZ) and phorbol 12-myristate 13-acetate (PMA). Values are means \pm SD. **Significantly different from each Pre value, $P < 0.01$.

0.01) on *day 1*, and remained increased until *day 3* (Table 1). IL-8 rose nonsignificantly at Post and Post 1 h on *day 1* but rose significantly at Post on *day 3* ($P < 0.05$) (Table 1). Cytokine response to each exercise session did not differ significantly among days.

Stress hormones. GH rose significantly during 30-min exercise ($P < 0.05$), during 60-min exercise, and Post ($P < 0.01$) but dropped dramatically Post 1 h and Post 3 h (Fig. 3). This dramatic change tended to decrease by daily repeated exercise sessions (*day 1* vs. *day 3*, $P = 0.088$). Exercise induced catecholamine responses, which were significant only during 60-min exercise (epinephrine: $P < 0.05$; norepinephrine: $P < 0.01$; dopamine: $P < 0.01$) on *day 1* (Fig. 4). Interestingly, exercise on *day 3* released quantities of catecholamines that far exceeded those released on *day 1*, and the magnitude of norepinephrine and dopamine responses rose significantly on *day 3* vs. *day 1* ($P < 0.01$)

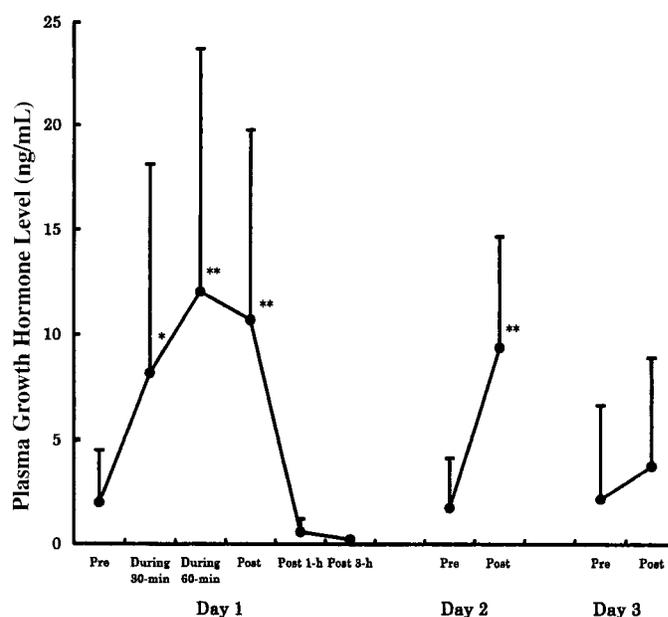


Fig. 3. Effects of 3 consecutive daily sessions of same-intensity endurance bicycle exercise (90 W, 90 min) on growth hormone dynamics. Values are means \pm SD. *Significantly different from each Pre value, $P < 0.05$; **Significantly different from each Pre value, $P < 0.01$.

(Fig. 4). Cortisol levels rose after exercise, but the increment was not significant (data not shown).

Serum biochemistry. Serum CK activity rose significantly after exercise sessions (delayed onset); it was only at Post 12 h that a significant increase ($P < 0.05$) was observed at 173 ± 100 IU/l compared with the Pre value of 134 ± 34 IU/l, and the level remained elevated for the experimental period and peaked to 352 ± 253 IU/l immediately after the third exercise session ($P < 0.01$). In contrast, serum Mb concentration rose dramatically not during 30- or 60-min exercise but at Post 1 h and Post 3 h ($P < 0.01$) and returned at Post 12 h (Fig. 5). It did not rise significantly, however, on *days 2* and *3* (Fig. 5). Serum C3 concentration did not decrease during 30- or 60-min exercise but did significantly ($P < 0.05$) at Post 1 h and Post 3 h (data not shown).

Interrelationships between bioactive substances and neutrophil responses. We attempted, first, to clarify the relationship of the dynamics of bioactive substances during exercise to neutrophil mobilization and priming (Table 2). AUC for IL-6 was positively correlated with a

Table 1. Effects of daily sessions of endurance bicycle exercise (90 W, 90 min) on circulating cytokine levels

	First Exercise Session							Third Exercise Session	
	Pre	30-min	60-min	Post	Post 1 h	Post 3 h	Post 12 h	Pre	Post
IL-1 β , pg/ml	12.7 \pm 13.3	9.7 \pm 11.4	9.4 \pm 14.5	9.6 \pm 13.3	11.9 \pm 13.4	7.8 \pm 9.1	10.9 \pm 11.8	8.7 \pm 5.5	13.3 \pm 11.0
IL-6, pg/ml	18.1 \pm 25.5	32.4 \pm 28.9	31.5 \pm 20.3	43.3 \pm 34.8*	30.5 \pm 27.6	57.1 \pm 38.8†	63.9 \pm 36.2†	44.6 \pm 36.5*	54.3 \pm 36.6*
IL-8, pg/ml	5.4 \pm 1.6	5.2 \pm 3.9	5.3 \pm 2.9	6.9 \pm 4.3	7.1 \pm 5.5	5.1 \pm 4.1	4.5 \pm 4.1	4.4 \pm 2.7	7.5 \pm 2.8†
TNF- α , pg/ml	19.2 \pm 9.5	16.4 \pm 11.5	26.0 \pm 23.6	22.3 \pm 11.5	19.5 \pm 9.9	16.6 \pm 10.1	18.2 \pm 9.1	18.7 \pm 9.5	20.8 \pm 9.0
IFN- γ , IU/ml	0.15 \pm 0.04	0.16 \pm 0.05	0.15 \pm 0.03	0.16 \pm 0.03	0.20 \pm 0.13	0.16 \pm 0.04	0.20 \pm 0.07	0.15 \pm 0.03	0.16 \pm 0.04

Values are means \pm SD. IL-1 β , -6, and -8: interleukin-1 β , -6, and -8, respectively; TNF- α , tumor necrosis factor- α ; IFN- γ , interferon- γ ; Pre, at rest before exercise; Post, after exercise. * $P < 0.05$. † $P < 0.01$ compared with Pre values of the first exercise session. ‡ $P < 0.05$ compared with Pre value of the third exercise session.

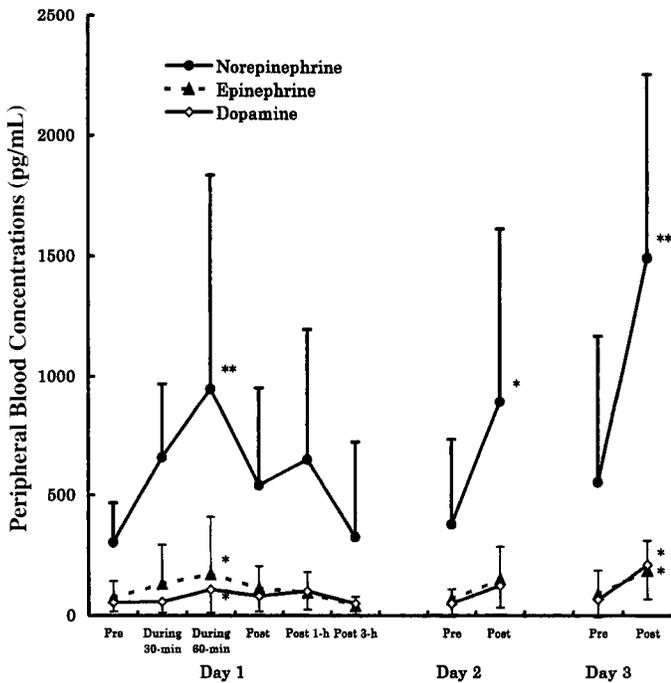


Fig. 4. Effects of 3 consecutive daily sessions of same-intensity endurance bicycle exercise (90 W, 90 min) on catecholamine responses. Values are means \pm SD. *Significantly different from each Pre value, $P < 0.05$; **Significantly different from each Pre value, $P < 0.01$.

percent increase in neutrophil count at Post and with the percent increases of neutrophil CL responses at Post and Post 1 h. AUC for IL-8 was correlated with the increments in the band neutrophil count and band neutrophil-to-total neutrophil ratio at Post. AUC for GH was also correlated positively with the increments of neutrophil count and PMA-stimulated CL response, but it was observed rather in a delayed-onset manner. AUC for cortisol was correlated with the percent rise in neutrophil count, especially with that of the band neutrophil count. On the other hand, some negative

correlations were seen between catecholamine responses and neutrophil dynamics.

Interrelationships between neutrophil responses and subsequent muscle damage. Next, we examined the interrelationship between neutrophil behavior and muscle damage (Table 2). Strong positive correlations were seen between Mb values at Post 1 h and the percent increment of neutrophil count and PMA-stimulated CL responses at Post. Mb values at Post 3 h were also correlated closely with the rise in neutrophil count at Post and PMA-stimulated CL response at Post and Post 1 h. Furthermore, CK response at Post 12 h was correlated with neutrophil dynamics, although the measurement points differed by ~ 10 h. Also, levels of CK at Post 12 h were closely correlated with those of Mb at Post 1 h ($r = 0.82$, $P < 0.05$) and Post 3 h ($r = 0.95$, $P < 0.01$).

Interrelationships between oxygen demand and the experimental variables. The $\% \dot{V}O_{2\max}$ values, which are the exercise intensity loaded for individual subjects, were correlated significantly with band neutrophil-to-total neutrophil ratio at Post ($r = 0.81$, $P < 0.05$) and Post 1 h ($r = 0.84$, $P < 0.01$), with band neutrophil-to-total leukocyte ratio at Post ($r = 0.85$, $P < 0.01$) and Post 1 h ($r = 0.80$, $P < 0.05$), and with the percent increase in band neutrophil count at Post ($r = 0.89$, $P < 0.01$) and Post 1 h ($r = 0.74$, $P < 0.05$). Interestingly, the mean $\dot{V}O_2$ per kilogram during exercise, which also reflects individual stress loading due to increased oxygen demand, was correlated with the AUC for GH ($r = 0.62$) and IL-6 ($r = 0.55$) and with the percent increase in PMA-stimulated CL response at Post 1 h ($r = 0.72$, $P < 0.05$).

DISCUSSION

Exercise is known to induce delayed-onset leukocytosis due to neutrophilia, depending on physical workload (11, 13, 16, 24, 25, 26b). We confirmed neutrophilia with a leftward shift as seen previously (11, 24) and a

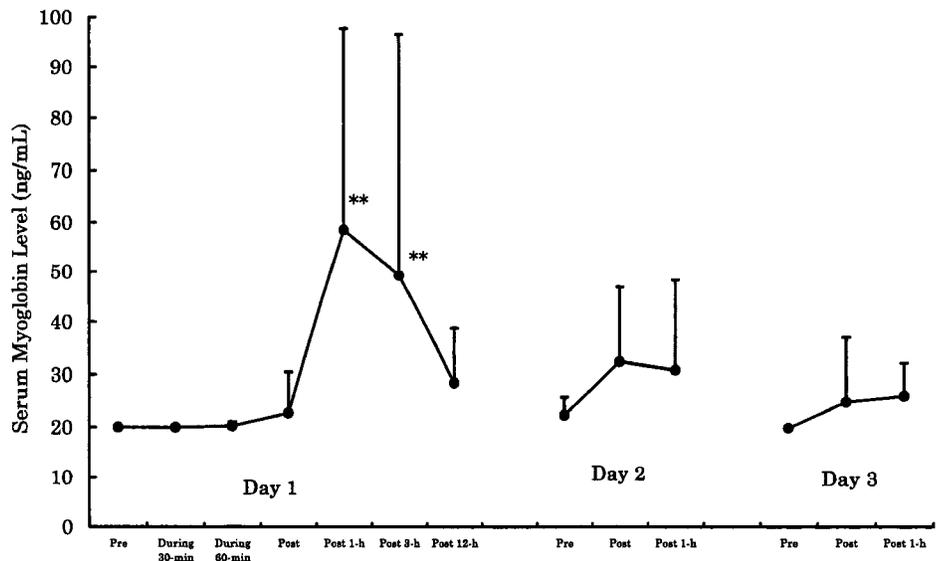


Fig. 5. Effects of 3 consecutive daily sessions of same-intensity endurance bicycle exercise (90 W, 90 min) on serum myoglobin (Mb) concentration as a marker for skeletal muscle damage. Values are means \pm SD. Lower sensitivity limit of Mb concentration was 20 ng/ml. **Significantly different from each Pre value, $P < 0.01$.

Table 2. Associations among exercise-induced responses of circulating bioactive substances, neutrophil behavior, and indexes for muscle damage assessed by correlation coefficient

	%Increases at Post Compared With Pre Values					%Increases at Post 1 h Compared With Pre Values				
	Neutrophil count	Band neutrophil count	Band neutrophil-to-total neutrophil ratio	OZ-CL	PMA-CL	Neutrophil count	Band neutrophil count	Band neutrophil-to-total neutrophil ratio	OZ-CL	PMA-CL
IL-6 AUC	0.77*		-0.45	0.56	0.88†				0.81*	0.82*
IL-8 AUC		0.58	0.74*					0.55		
GHAUC	0.60					0.85*	0.50			0.61
Cortisol AUC	0.50	0.62	0.40			0.54	0.50			
Epi AUC			-0.40		-0.55	-0.44				
NE AUC			-0.51		-0.52				-0.47	
Mb Post 1 h	0.86†				0.86†	0.48	0.43		0.60	0.58
Mb Post 3 h	0.85†				0.73*	0.63	0.51		0.44	0.78*
CK Post 12 h	0.76*				0.70	0.60	0.57			0.68

OZ-CL, opsonized zymosan-stimulated chemiluminescence response of neutrophils; PMA-CL, phorbol 12-myristate 13-acetate-stimulated chemiluminescence response of neutrophils; AUC, area under the curve calculated from Pre-to-Post-1-h data; GH, growth hormone; Epi, epinephrine; NE, norepinephrine; Mb, serum myoglobin concentration; CK, serum creatine kinase activity. Correlation coefficients of less than ± 0.4 were deleted as being not significant. * $P < 0.05$. † $P < 0.01$.

positive correlation between individual $\dot{V}O_{2\max}$ and the magnitude of neutrophilia, especially of band neutrophil mobilization, suggesting that stress workload may induce neutrophil mobilization from bone marrow reserve. The effect of shear force due to enhanced blood flow during exercise can be ruled out here because neutrophilia was rather accentuated after exercise ended. On the other hand, it has been pointed out that neutrophil mobilization is intermediated by exercise-intensity-dependent secretion of stress hormones such as catecholamine (25), cortisol (11, 16, 25, 26b), and GH (13, 26b). We found that the cortisol and GH responses were correlated positively with neutrophil mobilization. Especially, because of the similar time course of changes, GH was strongly suggested to be involved in delayed-onset neutrophilia. Also, IL-6 and IL-8 kinetics were correlated positively with the rise in neutrophil count and band neutrophil count with a leftward shift, respectively. These results indicate that stress hormones and circulating cytokines may be important for inducing neutrophilia.

PMN elastase, a powerful neural serine proteinase released from azurophilic granules of neutrophils, has been reported to be a parameter of prognostic significance in the development of fatal complications in severely ill patients (17). Because exhaustive exercise also raises the plasma level of neutrophil-derived granular enzymes, some researchers maintained that neutrophils were activated after exercise, because enzyme release was enhanced (8, 11). However, when data were adjusted per cell in our study, the possibility of enhanced degranulation was denied. We confirmed that the exercise-induced rise in PMN elastase was a concurrent phenomenon with substantial neutrophilia. It may be due to blood processing for plasma separation, inevitable in sampling, or in vivo hemodynamic stress just when neutrophilia occurred. Thus plasma PMN elastase is not a useful parameter for neutrophil "activation" in vivo when a similar extent of neutrophilia exists but should be useful when the rise exceeds that of neutrophil count in such situations as sepsis (17).

The luminol-dependent CL response of neutrophils largely measures myeloperoxidase-catalyzed production of highly toxic oxidants such as hypochlorous acid (12). We have reported that the OZ-stimulated CL response was significantly enhanced after exhaustive exercise of either high intensity (25) or longer duration exceeding 60 min (24, 26). In the present study, we looked for signal-transduction mechanisms (receptor mediated or not) by stimulating cells with different substances: OZ and PMA. OZ, a particular stimulant for phagocytosis, binds mainly to complement receptor type 3 (CR3, CD11b) on the cell surface and leads to phagocytosis, NADPH-oxidase activation, and degranulation (12). PMA is a soluble stimulant that activates NADPH-oxidase and degranulation by directly activating protein kinase C and distal signal transduction in place of diacylglycerol, through which receptor-mediated stages of functional modulation can be bypassed (12). We found that endurance exercise enhanced neutrophil CL response on stimulation with not only OZ but also PMA, but the former response was greater. These results suggest that the CR3 expression on neutrophils might be enhanced as previously reported (19) and that a certain signal transduction distal from protein kinase C was primed for enhanced activation.

As for mechanisms of neutrophil priming, we demonstrated close associations between IL-6 kinetics and the enhancement of neutrophil CL responses, suggesting that IL-6 may intermediate the functional modulation of neutrophils (20). Because no previous studies could reproduce the priming in vitro by prior exposure of standard intact neutrophils to plasma obtained after endurance exercise (22, 24), heterogeneous neutrophils mobilized after exercise are considered to be more sensitive to priming substances. Other inflammatory mediators causing neutrophil priming, such as anaphylatoxin C3a and chemoattractant C5a, have also been reported to rise after strenuous exercise (4, 6). Our study showed that serum C3 concentration decreased not during but 1 h after exercise, with the onset of the decrease coinciding with Mb release. These results

suggest that the endogenous complement was consumed to produce C3a and C5a, due possibly to muscle tissue damage, and that they may work not in the earlier phase but in the later phase of inflammatory induction.

Serum CK and Mb levels began to rise after, not during, the first exercise session, indicating that muscle damage depends on inflammation secondary to the mechanical rupture due to excessive repetition of muscle contraction. Although serum CK activity was correlated positively with Mb concentration, the pattern of the Mb response to acute single bouts of exercise was sharper and more sensitive than that of CK. Because the molecular mass of Mb (17 kDa) is much lower than that of CK (82 kDa), Mb leaks easily from damaged muscle and disappears sooner from the circulation due to renal excretion, resulting in sharper responses. We found strong associations between enhanced neutrophil responses and myocellular protein release, suggesting that systemic neutrophil hyperactivity may affect muscle inflammation at local levels after exercise. Because neutrophilia was sustained even after the end of exercise despite decreased microvascular blood flow in muscle, it may entrap primed neutrophils for transmigration into injured tissues or obstruct the microcirculation, resulting in ischemic injury. In any case, our data indicated that exercise-induced muscle damage might not be just a "local" event but one fairly subject to the regulation of "systemic" factors.

Although it has been reported that GH and IL-6 possess pharmacological action of mobilizing and priming neutrophils (2, 10, 13, 14, 20), this was adapted to in vivo situations such as an exercise-induced stress reaction, especially in subjects with increased oxygen demand. Because GH and IL-6 are released in response to stress unrelated to inflammation (7, 13), the release of these substances observed during and immediately after exercise was stress-related and indeed preceded the onset of muscle inflammation. Because GH appeared earlier than IL-6, GH may operate as a trigger of neutrophil mobilization and priming, and IL-6 augments the inflammatory process. On the other hand, the later increment of IL-6 coincided with myocellular protein release, suggesting that IL-6 was produced in the inflamed tissue and diffused into the circulation, as are Mb and CK. Because neutrophilia and enhanced oxidative capacity of neutrophils were closely correlated with, but appeared earlier than, myocellular protein release, it was suggested that neutrophils primed by these bioactive substances might be an important effector of exercise-induced muscle damage. Several clinical studies on SIRS also showed that IL-6 release was closely associated with upregulation of CR3 and enhanced luminol-dependent CL response of neutrophils, which well reflected the severity of illness and organ dysfunction (14, 20). Thus our study provided a good rationale that observed changes and their interactions are not an exercise-specific response but broadly relevant to SIRS.

Neutrophil dynamics and myocellular protein release gradually disappeared as daily repetition of exer-

cise progressed, as first demonstrated in a previous study from our laboratory (24, 29). This may be because the systemic release of the above bioactive substances was attenuated. In addition, we found that catecholamine release became more pronounced with progressive daily exercise. Because catecholamines inhibit neutrophil activity (15), the enhanced release may be an adaptive mechanism to suppress neutrophil priming while serving for energy production and maintenance of cardiopulmonary hyperdynamics during exercise. In view of the fact that exaggerated or prolonged systemic inflammatory response leads to organ dysfunction (1), the attenuated response of neutrophils to repeated sessions of endurance exercise can be an adaptation mechanism for preventing pathophysiological reactions. The balance and imbalance of a variety of endogenous agonistic and antagonistic factors might determine neutrophil dynamics and play an important role in coordinated regulation of systemic inflammatory response of the stressed body.

NOTE ADDED IN PROOF

Subsequent research in our laboratory on detailed profile of cytokine and hormonal changes after a marathon race and maximal exercise demonstrated that other bioactive substances, such as granulocyte colony-stimulating factor and prolactin, in addition to IL-6, IL-8, and GH significantly rose (26a, 26b). These substances might also contribute to the neutrophil mobilization and priming following exhaustive exercise.

Address for reprint requests and other correspondence: S. Nakaji, Dept. of Hygiene, Hirosaki Univ. School of Medicine, 5 Zaifu-cho, Hirosaki, Aomori 036-8562, Japan.

Received 30 November 1998; accepted in final form 1 June 1999.

REFERENCES

1. **Beal, A. L., and F. B. Cerra.** Multiple organ failure syndrome in the 1990s. Systemic inflammatory response and organ dysfunction. *JAMA* 271: 226–233, 1994.
2. **Borish, L., R. Rosenbaum, L. Albury, and S. Clark.** Activation of neutrophils by recombinant interleukin 6. *Cell. Immunol.* 121: 280–289, 1989.
3. **Cannon, J. G., W. J. Evans, V. A. Hughes, C. N. Meredith, and C. A. Dinarelli.** Physiological mechanisms contributing to increased interleukin-1 secretion. *J. Appl. Physiol.* 61: 1869–1874, 1986.
4. **Cannon, J. G., M. A. Fiatarone, R. A. Fielding, and W. J. Evans.** Aging and stress-induced changes in complement activation and neutrophil mobilization. *J. Appl. Physiol.* 76: 2616–2620, 1994.
5. **Cannon, J. G., S. N. Meydani, R. A. Fielding, M. A. Fiatarone, M. Meydani, M. Farhangmehr, S. F. Orencole, J. B. Blumberg, and W. J. Evans.** Acute phase response in exercise. Associations between vitamin E, cytokines, and muscle proteolysis. *Am. J. Physiol.* 260 (Regulatory Integrative Comp. Physiol. 29): R1235–R1240, 1991.
6. **Castell, L. M., J. R. Poortmans, R. Leclercq, M. Brasseu, J. Duchateau, and E. A. Newsholme.** Some aspects of the acute phase response after a marathon race, and the effects of glutamine supplementation. *Eur. J. Appl. Physiol.* 75: 47–53, 1997.
7. **Chrousos, G. P.** The hypothalamic-pituitary-adrenal axis and immune-mediated inflammation. *N. Engl. J. Med.* 332: 1351–1362, 1995.
8. **Dufaux, B., and U. Order.** Plasma elastase-1-antitrypsin, neutrophin, tumor necrosis factor, and soluble interleukin-2 recep-

- tor after prolonged exercise. *Int. J. Sports Med.* 10: 434–438, 1989.
9. **Fielding, R. A., T. J. Manfredi, W. Ding, M. A. Fiatarone, W. Evans, and J. G. Cannon.** Acute phase response in exercise. Neutrophil and IL-1 accumulation in skeletal muscle. *Am. J. Physiol.* 265 (*Regulatory Integrative Comp. Physiol.* 34): R166–R172, 1993.
 10. **Fu, Y. K., S. Arkins, G. Fuh, B. C. Cunningham, J. A. Wells, S. Fong, M. J. Cronin, R. Dantzer, and K. W. Kelley.** Growth hormone augments superoxide anion secretion of human neutrophils by binding to the prolactin receptor. *J. Clin. Invest.* 89: 451–457, 1992.
 11. **Hansen, J.-B., L. Wilsgard, and B. Osterud.** Biphasic changes in leukocytes induced by strenuous exercise. *Eur. J. Appl. Physiol.* 62: 157–161, 1991.
 12. **Hasegawa, H., K. Suzuki, S. Nakaji, and K. Sugawara.** Analysis and assessment of the capacity of neutrophils to produce reactive oxygen species in a 96-well microplate format using lucigenin- and luminol-dependent chemiluminescence. *J. Immunol. Methods* 210: 1–10, 1997.
 13. **Hoffman-Goetz, L., and B. K. Pedersen.** Exercise and the immune system: a model of the stress response? *Immunol. Today* 15: 382–387, 1994.
 14. **Jiang, W. G., M. C. A. Puntis, and M. B. Hallett.** Neutrophil priming by cytokines in patients with obstructive jaundice. *Surgery* 7: 281–289, 1994.
 15. **Nielson, C. P.** β -Adrenergic modulation of the polymorphonuclear leukocyte respiratory burst is dependent upon the mechanism of cell activation. *J. Immunol.* 139: 2392–2397, 1987.
 16. **Nieman, D. C., S. L. Nehlsen-Cannarella, O. R. Fagoaga, D. A. Henson, A. Utter, J. M. Davis, F. Williams, and D. E. Butterworth.** Effects of mode and carbohydrate on the granulocyte and monocyte response to intensive, prolonged exercise. *J. Appl. Physiol.* 84: 1252–1259, 1998.
 17. **Nuijens, J. H., J. J. Abbink, Y. T. Wachtfogel, R. W. Colman, A. J. M. Eerenberg, D. Dors, A. J. M. Kamp, R. J. M. Strack, L. G. Thijs, and C. E. Hack.** Plasma elastase α_1 -antitrypsin and lactoferrin in sepsis: Evidence for neutrophils as mediators in fatal sepsis. *J. Lab. Clin. Med.* 119: 159–168, 1992.
 18. **Papanicolaou, D. A., J. S. Petrides, C. Tsigos, S. Bina, K. T. Kalogeras, R. Wilder, P. W. Gold, P. A. Deuster, and G. P. Chrousos.** Exercise stimulates interleukin-6 secretion: inhibition by glucocorticoids and correlation with catecholamines. *Am. J. Physiol.* 271 (*Endocrinol. Metab.* 34): E601–E605, 1996.
 19. **Pizza, F. X., J. B. Mitchell, B. H. Davis, R. D. Starling, R. W. Holtz, and N. Bigelow.** Exercise-induced muscle damage: effect on circulating leukocyte and lymphocyte subsets. *Med. Sci. Sports Exerc.* 27: 363–370, 1995.
 20. **Rosenbloom, A. J., M. R. Pinsky, J. L. Bryant, A. Shin, T. Tran, and T. Whiteside.** Leukocyte activation in the peripheral blood of patients with cirrhosis of the liver and SIRS. Correlation with serum interleukin-6 levels and organ dysfunction. *JAMA* 274: 58–65, 1995.
 21. **Smith, J. A., R. D. Telford, M. S. Baker, A. J. Hapel, and M. J. Weidemann.** Cytokine immunoreactivity in plasma does not change after moderate endurance exercise. *J. Appl. Physiol.* 73: 1396–1401, 1992.
 22. **Smith, J. A., R. D. Telford, I. B. Mason, and M. J. Weidemann.** Exercise, training and neutrophil microbicidal activity. *Int. J. Sports Med.* 11: 179–187, 1990.
 24. **Suzuki, K., S. Naganuma, M. Totsuka, K.-J. Suzuki, M. Mochizuki, M. Shiraishi, S. Nakaji, and K. Sugawara.** Effects of exhaustive endurance exercise and its one-week daily repetition on neutrophil count and functional status in untrained men. *Int. J. Sports Med.* 17: 205–212, 1996.
 25. **Suzuki, K., H. Sato, T. Kikuchi, T. Abe, S. Nakaji, K. Sugawara, M. Totsuka, K. Sato, and K. Yamaya.** Capacity of circulating neutrophils to produce reactive oxygen species after exhaustive exercise. *J. Appl. Physiol.* 81: 1213–1222, 1996.
 26. **Suzuki, K., M. Totsuka, S. Naganuma, M. Shiraishi, M. Mochizuki, S. Nakaji, and K. Sugawara.** Effects of endurance exercise on blood neutrophils in sedentary men. *J. Phys. Fitness Nutr. Immunol.* 4: 18–24, 1994.
 - 26a. **Suzuki, K., M. Yamada, K. Kowatari, S. Kudoh, Q. Liu, S. Nakaji, K. Sugawara, S. Kurakake, N. Okamura, M. Totsuka, and K. Sato.** Effects of maximal exercise on plasma and urine cytokine concentrations in athletes. *J. Phys. Fitness Nutr. Immunol.* 8: 176–177, 1998.
 - 26b. **Suzuki, K., M. Yamada, S. Kurakake, N. Okamura, K. Yamaya, Q. Liu, S. Kudoh, K. Kowatari, S. Nakaji, and K. Sugawara.** Circulating cytokines and hormones with immunosuppressive but neutrophil priming potentials rise after endurance exercise in humans. *Eur. J. Appl. Physiol.* In press.
 27. **Thuma, J. R., R. Gilders, M. Verdun, and A. B. Loucks.** Circadian rhythm of cortisol confounds cortisol responses to exercise: implications for future research. *J. Appl. Physiol.* 78: 1657–1664, 1995.
 28. **Tidball, J. G.** Inflammatory cell response to acute muscle injury. *Med. Sci. Sports Exerc.* 27: 1022–1032, 1995.
 29. **Totsuka, M., S. Naganuma, K. Suzuki, S. Nakaji, S. Sato, and S. Sugawara.** Rapid adaptation of creatine kinase responses to repeated daily endurance exercise. *J. Phys. Fitness Nutr. Immunol.* 6: 187–190, 1996.