Exhaustive Exercise Induces Differential Changes in Serum Granulysin and Circulating Number of Natural Killer Cells

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The circulating number of natural killer (NK) cells largely changes after an acute bout of physical exercise. Granulysin is a cytolytic granule protein with a broad range of antimicrobial and tumoricidal activities produced and released by human NK cells and cytolytic T lymphocytes. Since NK cells constitutively produce granulysin, most serum granulysin in healthy humans is derived from NK cells. Serum granulysin levels in the healthy humans may therefore reflect the size of whole-body NK cell population in the body. The aim of this study was to determine the effect of an acute bout of exhaustive exercise on serum granulysin in comparison with the circulating number of NK cells. Six healthy, young male volunteers participated in the study. Each subject underwent both exhaustive exercise and resting sessions in a random order with at least a seven-day interval. Subjects were asked to run to exhaustion on a treadmill with an incremental graded protocol. Blood samples were collected before, immediately after, and 1 hr, 3 hr, 6 hr, 12 hr and 24 hr after exercise. Serum granulysin levels were measured by enzyme-linked immunosorbent assay (ELISA). NK cells were determined by flow cytometry. Exhaustive exercise induced a 4.8-fold increase in peripheral blood NK cells, but no significant change in serum granulysin. Our results support the hypothesis that exhaustive exercise-induced changes in the circulating number of NK cells represent a redistribution of lymphocytes, rather than the change in the size of whole-body NK cell population.

serum granulysin; natural killer cells; exhaustive exercise

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Granulysin is a cytolytic granule protein with a broad range of antimicrobial and tumoricidal activities produced by human natural killer (NK) cells and cytolytic T lymphocytes (CTL) (Pena and Krensky 1997; Pena et al. 1997; Stenger et al. 1998; Krensky 2000). Microarray analysis of mRNA isolated from human NK cells and CD8+ T cells confirmed that granulysin, perforin and
granzymes are all expressed in NK cells and activated CD8\(^+\) T cells (CTL) (Obata-Onai et al. 2002). Flow cytometric analysis showed that most of CD3\(^-\)CD56\(^+\) NK cells intensely express intracellular granulysin, which is also expressed in a small portion of CD3\(^+\) T cells but not in other cell types, including B cells, monocytes and granulocytes in peripheral blood (Ogawa et al. 2003).

Recently, we developed an enzyme-linked immunosorbent assay (ELISA) system for detecting granulysin in sera or culture supernatants. Interestingly, we found that granulysin is spontaneously secreted by peripheral blood NK cells. In immunodeficiency patients lacking NK cells, serum granulysin was almost undetectable, but gradual increases of serum granulysin levels were observed after bone marrow transplantation concomitant with the recovery of NK cells (Ogawa et al. 2003). Since NK cells but not unstimulated CTL secrete granulysin constitutively, most serum granulysin is considered to be produced by NK cells in healthy humans (Obata-Onai et al. 2002; Ogawa et al. 2003). Therefore, the level of serum granulysin in healthy humans may reflect the size of whole body NK cell population.

It is well-known that both the circulating number and cytolytic activity of NK cells largely change after an acute bout of physical exercise in humans (Deuster et al. 1988; Shinkai et al. 1992; Nieman et al. 1993; Nielsen et al. 1996, 1998; Woods et al. 1998; van der Pompe et al. 2001). Several studies have suggested that the increase or the decrease in blood NK cell activity can be ascribed to numerical shifts in blood NK cells but not change in the activity level of NK cells on a per cell basis (Shinkai et al. 1992; Nieman et al. 1993; Miles et al. 2002). Whether such numerical shifts in blood NK cell numbers induced by exercise correspond to the changes in the size of whole body NK cell population has not been investigated.

We hypothesized if exercise-induced changes in peripheral blood NK cell numbers reflect the change in the size of whole body NK cell population, the level of serum granulysin should change in response to exercise. The aim of this study, therefore, was to determine the effect of an acute bout of exhaustive exercise on serum granulysin levels in comparison with the circulating number of peripheral blood NK cells.

**Materials and Methods**

**Subjects**

Six healthy, young male volunteers (age 22.5 ± 0.6 years, height 172.9 ± 1.5 cm, weight 65.5 ± 2.2 kg) participated in the exhaustive treadmill exercise experiment. All subjects were carefully instructed about the experimental procedures and potential risks, and all gave their informed consent. All experimental procedures were carried out under the approval of the ethical committee of Tohoku University.

**Experimental design and blood sampling**

Each subject completed two trial conditions in a random order: exhaustive treadmill exercise (EXERCISE) and time-matched sedentary control (CONTROL) with at least seven days of sedentary life without any type of extra exercise other than daily physical activity between two conditions.

Exhaustive treadmill exercise was performed by an incremental graded protocol that was finished within 30 min. Subjects were asked to exercise to exhaustion. Heart rate was monitored continuously with Polar Vantage NV Heart Rate Monitor (Polar Electro, Kempele, Finland). Subjects’ expiratory gases were analyzed by AeroMonitor (AE-280; Minato Medical Science Co., Osaka). On arrival at the laboratory at 8:30 AM, subjects rested in a sitting position for 30 min, and a venous catheter (Terumo, Tokyo) was inserted into a forearm superficial vein. Resting blood samples (before exercise) were obtained at 9:00 AM. They started the exercise at 10:00 AM. Blood samples were drawn before exercise (Pre), immediately after exhaustion (Post), 1 hr, 3 hr, 6 hr, 12 hr and 24 hr after exhaustive exercise. For the CONTROL trial, subjects rested quietly in the laboratory, and blood samples were taken at time points corresponding to the sampling times in the EXERCISE trial.

**Leukocyte counts and subset analysis**

Complete blood count (CBC) analysis was performed using an automated hematology analyzer (Sysmex pocH-100i, Sysmex Corporation, Kobe). Leukocyte differentials were determined by May-Grunwald/Giemsa staining (MERCK, Tokyo). Cells were identified as lymphocytes, monocytes, neutrophils, eosinophils and baso-
phils by standard morphology, and at least 400 cells were counted under ×400 magnification. The percentage and absolute numbers of each cell type were calculated. Changes in plasma volume were calculated from the measurements of hemoglobin and hematocrit according to the method described by Dill and Costill (1974). Leukocyte total and subset counts were adjusted for shifts in plasma volume.

Flow cytometry

A volume of 100 μl of EDTA-treated whole blood was incubated with mouse serum (Sigma, St. Louis, MO, USA) for 30 min at room temperature to block nonspecific antibody binding via IgG Fc receptors and then mixed with anti-human monoclonal antibodies (mAb) in the following staining combinations: FITC-labeled anti-CD56 mAb (NCAM16.2)/PE-labeled anti-CD3 mAb (UCHT1); and FITC-labeled anti-CD4 mAb (RPA-T4)/PE-labeled anti-CD8 mAb (SK1)/PerCP-Cy5.5-labeled anti-CD3 mAb (SK7). All mAb were purchased from BD Biosciences (San Jose, CA, USA). After 30-min incubation in the dark, red blood cells were lysed with FACS lysing solution (BD Biosciences). Non-lysed cells were centrifuged and then washed twice with cold phosphate buffered saline (PBS). The stained cells were analyzed on a FACScan flow cytometer using CELLQuest software (BD Biosciences). The lymphocytes were gated on the basis of forward vs. side scatter profiles.

The FACScan indicates the percentage of cells bearing the specific fluorescence label within the gated lymphocyte region. Absolute cell counts were derived by multiplying the percentage of a given cell subset by the total lymphocyte concentration found in peripheral blood. All lymphocyte subset counts were adjusted for shifts in plasma volume.

Granulysin quantification by ELISA

The collected blood samples were stored on ice until centrifugation. Serum samples were obtained by centrifugation of the whole blood sample at 3,000 rpm for 10 min at 4°C and frozen at -80°C until analysis. Serum granulysin levels were measured by ELISA as described previously (Ogawa et al. 2003). Briefly, anti-granulysin mAb RB1 was coated on microtiter plates (Nunc, Roskilde, Denmark) at 5 μg/ml in 100 mM carbonate buffer at 4°C overnight. The plates were washed with PBS containing 0.1% Tween-20 (washing buffer) and blocked with 10% fetal bovine serum (FBS) in washing buffer (blocking buffer) at 37°C for 1-2 hr. Then, the plates were serially reacted at room temperature with the following materials with washing steps between each reaction; samples or standards in blocking buffer for 2 hr, 0.1 μg/ml of biotinylated RC8 mAb in blocking buffer for 1 hr, and 0.05 U/ml of β-galactosidase-conjugated streptavidin (Roche Diagnostics) in washing buffer for 1 hr. After washing, the plates were finally incubated with 0.4 mM of 4-methylumbelliferyl-β-D-galactoside (Sigma) in 10 mM sodium phosphate buffer (pH 7.0) containing 0.02% BSA, 100 mM NaCl, and 1 mM MgCl₂ at 37°C for 17 hrs. Then, the enzyme reaction was stopped with 100 mM glycine-NaOH (pH 10.3), and the fluorescence intensity was measured with CytoFluor 4000 fluorescence multi-well plate reader (Applied Biosystems, Foster City, CA, USA) with excitation and emission wavelength of 360 nm and 460 nm, respectively.

Standard granulysin was purified partially from the serumfree culture supernatant of pGrn-FLAG-transfected COS-7 cells by affinity chromatography with heparin Sepharose CL-6B (Amersham Biosciences) followed by gel filtration with Superdex-75 (Amersham Biosciences). Purity of granulysin in the standard sample was determined by SDS-PAGE followed by Coomassie Brilliant Blue staining, and the gel image analysis performed with Intelligent Quantifier densitometer (Bio Image, Ann Arbor, MI, USA). Concentration of granulysin in the standard sample was calculated from the purity and the total protein concentration.

Hormone analyses

Venous blood samples for catecholamines and cortisol evaluation were distributed into a vacutainer collection tube (Terumo, Tokyo) containing EDTA immediately after sampling. After centrifugation at 3000 rpm for 10 min at 4°C, plasma samples were collected and frozen at -80°C until analysis. Plasma catecholamines including epinephrine, norepinephrine and dopamine were measured by high-performance liquid chromatography (HPLC) (Yoshimura et al. 1993). Plasma cortisol concentration was measured by radioimmunoassay (RIA) (Immunetech, Marseille, France), according to the manufacturer’s instructions. All parameters were adjusted for shifts in plasma volume.

Statistical analysis

Data were expressed as mean ± S.E. Catecholamines were analyzed using 2 (control and exercise conditions) × 6 (Pre, Post, 1 hr, 3 hr, 6 hr and 24 hr time points)
repeated-measures ANOVA. The other variables were analyzed using 2 (control and exercise conditions) × 7 (Pre, Post, 1, 3, 6, 12 and 24 hrs time points) repeated-measures ANOVA. Fisher’s PLSD post hoc test was used to locate differences when the ANOVA revealed a significant interaction. All statistical analyses were performed with StatView software (SAS Institute Inc., Cary, NC, USA). \( P < 0.05 \) was regarded as statistically significant.

**RESULTS**

**Exercise**

The average time to exhaustion was 19.6 ± 2.4 min. Peak oxygen uptake (\( \text{VO}_{2\text{peak}} \)) was 44.5 ± 3.9 ml·kg\(^{-1}\)·min\(^{-1}\). Peak heart rate (\( \text{HR}_{\text{peak}} \)) was 187 ± 3 beats·min\(^{-1}\). Plasma volume decreased (-9.58 ± 1.93%) immediately after exhaustive exercise, and returned to baseline 1 hr after exhaustion.

**Effect of exhaustive treadmill exercise on plasma catecholamines and cortisol**

Exercise resulted in a significant activation of the sympathetic nervous system (SNS), as indicated by elevated circulating catecholamine levels. Exhaustive exercise induced significant elevations in the concentrations of plasma epinephrine, norepinephrine and dopamine (Fig. 1A, B and C). Plasma epinephrine, norepinephrine and dopamine were remarkably increased immediately after exercise (7.75-, 5.96- and 4.07-fold of basal level, respectively) and returned to basal levels 1 hr after exhaustion. However, no significant change was observed in plasma cortisol levels (data not shown).

**Effect of exhaustive treadmill exercise on circulating numbers of leukocytes and lymphocytes**

Exhaustive exercise induced significant changes in the circulating leukocyte and lymphocyte counts (Fig. 2A and B). Leukocytes markedly increased immediately after exercise (1.58-fold of basal level), and returned to basal level 12 hrs after exhaustion. Lymphocytes markedly increased immediately after exercise (1.94-fold of basal level), but dropped below baseline 1 h after exhaustion (0.85-fold of baseline).

**Effect of exhaustive treadmill exercise on circulating numbers of NK, NKT and T cells**

Exhaustive exercise induced significant changes in the circulating numbers of \( \text{CD}^3^\text{−} \text{CD}56^+ \) NK, \( \text{CD}^3^\text{+} \text{CD}56^+ \) NKT and \( \text{CD}^3^\text{+} \text{CD}56^+ \) T cells (Fig. 3A, B and C). NK,
NKT and T cells were remarkably increased immediately after exercise (4.81-, 2.46- and 1.48-fold of the basal level, respectively), and dropped below baseline 1 hr after exhaustion (0.57-, 0.66- and 0.93-fold of baseline, respectively).

**Effect of exhaustive treadmill exercise on circulating numbers of CD3⁺CD4⁺, CD3⁺CD8⁺ T cells and CD4/CD8 ratio**

Exhaustive exercise induced significant changes in the circulating number of CD3⁺CD8⁺ T cells, but no changes in CD3⁺CD4⁺ T cells (Fig. 4A and B). CD3⁺CD8⁺ T cells were increased immediately after exercise (1.73-fold of baseline) and dropped below baseline 1 hr after exhaustion (0.73-fold of basal level). Exhaustive exercise also induced a significant change in the CD4/CD8 ratio (Fig. 4C). The CD4/CD8 ratio dropped below baseline immediately after exercise (0.69-fold of baseline) and returned to baseline 1 hr after exhaustion.

**Effect of exhaustive treadmill exercise on serum level of granulysin**

The exhaustive exercise did not induce significant change in serum granulysin levels (Fig. 5).
In this study we first confirmed the differential effect of exhaustive exercise on the serum levels of granulysin and NK cells. Our results show that there was no significant change in the level of granulysin after exhaustive exercise, whereas the number of NK cells increased about five-fold and subsequently decreased below baseline. Thus, exhaustive exercise, despite a large increase and a decrease thereafter in the circulating number of NK cells, did not substantially affect the size of whole body NK cell population.

Serum levels of granulysin are elevated in certain pathological conditions, acute viral infection (Ogawa et al. 2003) and pre-eclampsia (Sakai et al. 2004). In both viral infection and pre-eclampsia, NK cells and CTL were activated. Viral infection may also involve activation of NKT cells, and granulysin was expressed in activated NKT cells (Obata-Onai et al. 2002; Gansert et al. 2003). Elevation of serum granulysin level during acute viral infection or pre-eclampsia, therefore, may have reflected the increased secretion of granulysin from not only activated NK cells but also activated CTL. Because exhaustive exercise did not induce significant changes in the level of serum granulysin, exhaustive exercise had little effect on the activation of NK, NKT and CD8^+^ T cells.

β-adrenoceptor stimulation by catecholamines causes downregulated attachment of NK cells from cultured vascular endothelial cells (Benschop et al. 1994). Therefore the increase in human circulating NK cells by exercise or stressors is suggested to be mainly mediated via β-adrenoceptors (Schedlowski et al. 1996). Because catecholamines were markedly elevated during exhaustive exercise in this study as well as in the previous studies (Holmqvist et al. 1986; Field et al. 1991), the increase in the number of circulat-
ing NK cells was likely to be caused by catecholamine-induced transient detachment of NK cells from the vascular bed into circulation. Therefore, such changes may not affect the size of whole body NK cell population, despite a large fluctuation in the circulating number of NK cells.

We also observed differential changes among the circulating numbers of lymphocyte subsets in response to exhaustive exercise. NK cells were the most sensitive, followed by NKT cells, CD8+ cells and CD4+ cells. Most of the lymphocyte subsets bear β-adrenergic receptors, but the difference in the level of expression may have lead to the differential changes in the number of lymphocyte subsets in response to intense exercise. NK cells bear more β-adrenergic receptors than T cells, and cytotoxic T cells express more β-adrenergic receptors than helper T cells (Maisel et al. 1990; Landmann 1992). This may explain why NK cells are more sensitive to exhaustive exercise than other lymphocyte subsets.

It has been reported that the granulysin is spontaneously secreted by NK cells and CTL via a non-exocytotic pathway, and its release increases through the granule exocytosis pathway (Ogawa et al. 2003). What signal pathway is involved in the spontaneous release of granulysin is still under investigation, but our data suggest that β-adrenergic stimulation is not involved in the release of granulysin nor activation of NK cells and CTL. The limitation of this study was the small sample size. With 6 subjects small changes in granulysin could have been overlooked.

In summary, we first confirmed that level of serum granulysin remained stable, although the circulating number of NK cells was markedly increased during strenuous exercise. Our results support the hypothesis that exhaustive exercise-induced changes in the circulating number of NK cells represent redistribution of NK cells, rather than a fluctuation of the size of whole-body NK cell population or the activation of NK cells.

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References


Immunol., 33, 1925-1933.