Mobilization of NK cells by exercise: downmodulation of adhesion molecules on NK cells by catecholamines

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Nagao, Fumiko, Masatoshi Suzui, Kazuyoshi Takeda, Hideo Yagita, and Ko Okumura. Mobilization of NK cells by exercise: downmodulation of adhesion molecules on NK cells by catecholamines. Am J Physiol Regulatory Integrative Comp Physiol 279: R1251–R1256, 2000.—The change of plasma catecholamine concentration correlates with the change of natural killer (NK) activity and NK cell number in peripheral blood mononuclear cells (PBMC) during and after moderate exercise. We studied the causal relation between exercise-induced catecholamine and expression of adhesion molecules on NK cells during and after exercise. The expression of CD44 and CD18 on CD3+CD56+ NK cells was significantly reduced during exercise (P < 0.01). When PBMC were stimulated with 10^{-5} M norepinephrine in vitro, the expression of these adhesion molecules on CD3+CD56+ NK cells was downmodulated within 30 min. The binding capacity of NK cells to a CD44 ligand, hyaluronate, was reduced by the stimulation with norepinephrine (P < 0.01). The intravenous injection of norepinephrine in mice decreased the expression of CD44 and CD18 on CD3+CD56+ NK1.1+ cells (P < 0.01) and increased the number of CD3+CD56+ NK1.1+ cells in PBMC (P < 0.01). These findings suggest that exercise-induced catecholamines modulate the expression of adhesion molecules on NK cells, resulting in the mobilization of NK cells into the circulation.

NK cells, which might modulate the expression level of adhesion molecules critical for migration of NK cells.

METHODS

Subjects

Eight healthy, nonsmoking males (19.6 ± 0.5 yr old, maximal oxygen consumption = 47.8 ± 6.6 ml·kg^{-1}·min^{-1}) were used in this study. Exercise was performed with a cycle ergometer for 30 min at 110% of ventilation threshold (VT) V_{O2}. Blood samples were obtained 10 min before, immediately after, and 5, 15, 30, and 60 min after exercise for determination of NK activity, CD3+CD16+CD56+ NK cell number, and plasma epinephrine, norepinephrine, dopamine, β-endorphin, PGE₂, and cortisol concentrations.

NK Activity

Isolation of peripheral blood mononuclear cells. Peripheral blood mononuclear cells (PBMC) were separated by Ficoll-Hypaque density-gradient centrifugation, washed twice with PBS, and suspended in RPMI 1640 medium containing 10% (vol/vol) fetal bovine serum and penicillin-streptomycin (complete medium).

Cell lines. The K562 erythroleukemic cell line was used as a target cell in the assay. The cells were maintained in the complete medium and used in the early and mid-log phases for NK assay.

Labeling of target cells with europium. K562 target cells were labeled with nonradioactive europium-diethylentriaminepentaacetic acid (Eu-DTPA). The labeling procedure used was the same as previously described (4, 13). Before labeling, K562 cells were washed twice with buffer A (in mM: 50 HEPES, 93 NaCl, 5 KCl, and 2 MgCl₂). K562 cells were then incubated in a labeling buffer (in mM: 40 Eu, 125 DTPA, and 250 dextran sulfate) for 20 min at 4°C. After labeling, the cells were washed seven times with buffer B (in mM: 50...
HEPES, 93 NaCl, 5 KCl, 2 MgCl2, 2 CaCl2, and 10 dextrose) and twice with the complete medium and resuspended at a concentration of 1 × 10⁶ cells/ml.

**NK assay.** One hundred microliters of target cells labeled with Eu-DTPA were pipetted into the wells of a 96-well round-bottom microplate. An equal volume of effector cells was added to give effector-to-target cell ratios of 5:1, 10:1, and 20:1. The plate was centrifuged for 1 min at 1,000 g and then incubated for 2 h in a humidified 5% CO₂ atmosphere at 37°C. Twenty microliters of supernatant and one hundred microliters of enhancement solution (Pharmacia) were added into each well of a 96-well flat-bottom microplate (immunoassay plate, Nunc). The released Eu³⁺ was detected by measuring the fluorescence on a time-resolved fluorometer (Arcus 1232 Delphi fluorometer, Pharmacia). The spontaneous release was determined from the well with target cells alone. The maximum release was determined by lysing the target cells with 10 µl of 10% Triton X-100. The percentage of the Eu³⁺ release was calculated according to the formula

\[
\text{spontaneous release} = \frac{\text{maximum release} - \text{spontaneous release}}{\text{maximum release}} \times 100
\]

**Phenotypic Analyses**

Aliquots (1 × 10⁶) of PBMC were stained with FITC-labeled anti-CD16 (Leu-11a, Becton Dickinson, San Jose, CA), phycoerythrin (PE)-labeled anti-CD56 (NKH1, Coulter), and perpridinin-chlorophyll (PerCP)-labeled anti-CD3 (Leu-4, Becton Dickinson) monoclonal antibodies (MAbs) for 15 min at 4°C. After being washed twice with PBS, the cells were subjected to three-color flow cytometric analysis on a FACScan (Becton Dickinson). NK cell number in PBMC was determined as a percentage of CD3⁺ CD16⁺ CD56⁺ cells.

**Hormonal Measurement**

Epinephrine, norepinephrine, and dopamine in plasma were measured by HPLC. Plasma prostaglandin E₂, cortisol, and β-endorphin concentrations were determined by RIA.

**Expression of Adhesion Molecules**

FITC-labeled MAbs against CD31, CD44, CD49d, CD18, and CD62L were obtained from PharMingen (San Diego, CA). Aliquots of cells (1 × 10⁶) were stained with one of these FITC-labeled MAbs, PE-labeled anti-CD56 MAb, and PerCP-labeled anti-CD3 MAb at a concentration of 1 µg/100 µl for 15 min at 4°C. After being washed twice with PBS, the cells were subjected to three-color flow cytometric analysis on a FACScan. The expression levels of each adhesion molecule were determined by mean fluorescence intensity (MFI) of FITC fluorescence on CD3⁺ CD56⁺ cells.

**Binding Assay**

Coating. Tissue culture dishes (Becton Dickinson) were coated with 0.1% sodium hyaluronate (Wako, Osaka, Japan) in PBS for 1 h at 37°C. Control dishes were incubated with PBS for 1 h at 37°C.

**Stimulation of PBMC with norepinephrine.** PBMC were resuspended at 1 × 10⁶/ml in RPMI 1640 medium containing 1% BSA. After 10 µM norepinephrine was added to 5 ml of the PBMC suspension, the mixture was incubated for 30 min at 37°C. Another 5 ml of PBMC suspension did not receive norepinephrine as a control and was similarly incubated at 37°C for 30 min.

**Binding assay procedure.** After precoated dishes were washed twice with PBS, PBMC incubated with or without norepinephrine were added to the dishes. The cells were incubated to adhere for 1 h in a humidified 5% CO₂ atmosphere at 37°C. Nonadherent cells were carefully removed with a pipette after gentle agitation. The plates were then gently washed three times with prewarmed RPMI 1640 containing 1% BSA, and the remaining nonadherent cells were added to the former nonadherent cells. Adherent cells were then detached from the plates using Cell Scraper L (Sumilon, Tokyo, Japan) and collected with a pipette. After cells were counted, the nonadherent or adherent cells were stained with FITC-labeled anti-CD14 MAb (PharMingen), PE-labeled anti-CD56 MAb, and PerCP-labeled anti-CD3 mAb. The content of CD3⁺ CD14⁺ CD56⁺ NK cells was then analyzed by flow cytometry. The percent adherence of NK cells was calculated as

\[
\frac{\text{number of adherent NK cells}}{\text{sum of adherent and nonadherent NK cells}} \times 100
\]

**In Vivo Analyses in Mice**

**Experimental design.** Six-week-old male C57BL/6 mice were purchased from Charles River (Shizuoka, Japan). Five mice in each group were intravenously injected with 3 ng of norepinephrine in 100 µl of PBS or with PBS alone. After 10 min, PBMC were prepared from each mouse and analyzed for the frequency of NK cells and expression of CD44 and CD18 on NK cells.

**Isolation of PBMC.** PBMC were isolated by density-gradient centrifugation by using mouse-sodium meharizoate ficoll (M-SMF, JIMRD, Gunma, Japan), washed twice, and suspended in the complete medium.

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Fig. 1. Changes in natural killer (NK) activity and NK cell frequency in peripheral blood mononuclear cells (PBMC) during and after exercise. PBMC were prepared from venous blood drawn at the indicated time points before and after exercise. NK activity (A) was tested against K562 target cells at an effector-to-target cell ratio of 20 by the Eu release assay. Frequency of CD3⁺ CD16⁺ CD56⁺ NK cells was determined by flow cytometry. Data are means ± SD of 8 individuals. *P < 0.05, **P < 0.01 vs. before exercise.
Phenotypic analysis. Aliquots (1 × 10^6) of PBMC were stained with FITC-labeled anti-CD3 (145–2C11, PharMingen) and PE-labeled anti-NK1.1 (PK136, PharMingen) MAbs for 15 min at 4°C. After being washed twice with PBS, the cells were analyzed for frequency of CD3^−NK1.1^ cells by flow cytometry.

Expression of CD44 and CD18 on NK cells. Aliquots (1 × 10^6) of PBMC were stained with FITC-labeled anti-CD44 (IM7, PharMingen) or FITC-labeled anti-CD18 (C71/16, PharMingen) MAb, PE-labeled anti-NK1.1 MAb, and cytochrome-labeled anti-CD3 MAb (145–2C11, PharMingen) for 15 min at 4°C. The cells were then analyzed by three-color flow cytometry. The expression levels of CD44 and CD18 were determined by MFI of FITC fluorescence on CD3^−NK1.1^ cells.

Statistical Analyses

Data from the exercise experiments in vivo were analyzed using one-factor ANOVA. When a significant F ratio was demonstrated, differences between time points were determined by Bonferroni post hoc analysis. Student’s t-tests were carried out for the data from the experiments in vitro and in vivo for mice. Linear regressions were calculated by Pearson’s method. All statistical calculations were performed using StatView program for Macintosh (Abacus Concepts, Berkeley, CA), with statistical significance set at P < 0.05.

RESULTS

PBMC were prepared from venous blood drawn at 10 min before and 0, 10, 20, 30, and 60 min after an exercise (110% VT). NK activity was tested against K562 target cells by the Eu release assay, and the frequency of CD3^−CD16^CD56^ NK cells was determined by flow cytometry. Figure 1 represents means ± SD of eight individuals tested. NK activity was significantly increased during exercise (P < 0.01) and then suppressed after exercise (P < 0.05). The change in NK activity (Fig. 1A) was well correlated with the change in frequency of CD3^−CD16^CD56^ NK cells in PBMC (Fig. 1B) (r > 0.623 at 6 different time points, P < 0.05), suggesting that the change in NK activity of
PBMC during and after the exercise is predominantly determined by the NK cell contents in PBMC.

Simultaneously, we measured the blood concentrations of epinephrine and norepinephrine (Fig. 2). Dopamine, cortisol, β-endorphin, and PGE₂ were also measured, because these hormones have been known to affect NK activity (6, 7, 10, 17). The concentrations of epinephrine, norepinephrine, and dopamine were significantly increased during exercise (P < 0.001 or P < 0.01) and returned to basal levels after exercise. These transitions correlated closely with the change in NK activity (r = 0.624 at 4 time points and r = 0.320 at another 2 time points, P = 0.05) and the frequency of NK cells (r = 0.301 at 4 time points, P = 0.05). On the other hand, the concentrations of cortisol, β-endorphin, and PGE₂ did not change.

We also examined the changes in expression of various adhesion molecules (CD31, CD44, CD49d, CD18, and CD62L) on CD₃⁻CD₁₆⁻CD₅₆⁺ NK cells during and after exercise (Fig. 3). The expression of CD44 and CD18 was significantly decreased during exercise. The change in CD44 and CD62L expression on NK cells during the time course showed a reverse pattern to the NK activity and the frequency of NK cells. On the other hand, the expression of CD31 or CD49d on CD₃⁻CD₁₆⁻CD₅₆⁺ NK cells did not change.

We next examined the effect of catecholamines on the expression of these adhesion molecules on NK cells in vitro. PBMC were incubated with 10⁻⁸ M (1.69 ng/ml) norepinephrine for 10, 30, or 60 min at 37°C, and the expression of CD44, CD18, CD31, and CD49d on CD₃⁻CD₁₆⁻CD₅₆⁺ NK cells was determined by flow cytometry. The thin lines indicate CD44, CD18, CD31, and CD49d expression before stimulation. Similar results were obtained in 5 independent experiments.

Fig. 4. Effect of norepinephrine on the expression of CD44, CD18, CD31, and CD49d on NK cells in vitro. PBMC were stimulated with 10 μM norepinephrine for 10, 30, or 60 min at 37°C, and expression of CD44, CD18, CD31, and CD49d on CD₃⁻CD₁₆⁻CD₅₆⁺ NK cells (thick lines) was determined by flow cytometry. The thin lines indicate CD44, CD18, CD31, and CD49d expression before stimulation. Similar results were obtained in 5 independent experiments.

Fig. 5. Effect of norepinephrine on the binding of NK cells to hyaluronate. PBMC were prepared with or without 10 μM norepinephrine (NE) for 30 min before adherence on hyaluronic acid (HA)-coated or uncoated dishes for 1 h. Frequencies of CD₃⁻CD₁₆⁻NK cells in nonadherent and adherent cells were determined by flow cytometry, and % adherence of NK cells was calculated as described in METHODS. Data are means ± SD of triplicate dishes. Similar results were obtained in 5 independent experiments. **P < 0.01.

Fig. 6. Effect of norepinephrine on mobilization of NK cells in vivo. PBMC were prepared from C57BL/6 mice 10 min after iv injection of 3 ng of norepinephrine or PBS (control). Frequencies of CD₃⁻NK1.1⁺ NK cells (A) and expression of CD44 or CD18 on CD₃⁻NK1.1⁺ NK cells (B) were determined by flow cytometry. Bars represent means in each group; **P < 0.01. Similar results were obtained in 5 independent experiments.
It is known that the CD44 molecules on lymphocytes bind to hyaluronic acid on blood vessels. We next examined the effect of catecholamines on binding of NK cells to hyaluronic acid. After PBMC were stimulated with $10^{-6}$ M norepinephrine at 37°C for 30 min, the binding of CD3^+ CD56^+ NK cells to hyaluronic acid-coated plates was measured. As shown in Fig. 5, NK cells exhibited significantly higher binding to hyaluronate-coated plates than to uncoated plates ($P < 0.01$) in the absence of norepinephrine. However, in the presence of norepinephrine, the binding of NK cells to hyaluronate-coated plates was significantly inhibited ($P < 0.01$). These results suggested that norepinephrine inhibits binding of NK cells to hyaluronic acid by downmodulating CD44.

Finally, we verified the in vivo effect of catecholamines on the mobilization of NK cells and the expression of adhesion molecules on NK cells in the murine system. Mice were intravenously injected with 3 ng of norepinephrine per mouse, and the peripheral blood was taken after 10 min. The frequency of CD3^+ NK1.1^+ NK cells in PBMC and the expression of CD44 and CD18 on NK cells were analyzed by flow cytometry. As shown in Fig. 6A, the frequency of CD3^+ NK1.1^+ NK cells in PBMC was significantly increased by norepinephrine administration ($P < 0.01$). Concomitantly, the expression of CD44 and CD18 on CD3^+ NK1.1^+ NK cells was significantly decreased in norepinephrine-treated mice ($P < 0.01$; Fig. 6B).

**DISCUSSION**

In this study, we demonstrated that the dynamic change in peripheral blood NK activity during and after an exercise is associated with mobilization of NK cells, which appears to result from modulation of adhesion molecules such as CD44 on NK cells by exercise-induced catecholamines. In support of this notion, an intravenous injection of norepinephrine into mice induced a rapid mobilization of NK cells into circulation in association with downmodulation of adhesion molecules on NK cells.

Some previous studies have characterized the effect of catecholamines on mobilization of NK cells. Schedlovski et al. (21, 22) reported that catecholamines modulate human NK cell circulation and function by β-adrenergic mechanisms. They showed that NK cell numbers in PBMC were increased 6 times by epinephrine infusion and 1.3 times by norepinephrine infusion. Although the increased serum catecholamines during exercise seem to be mainly derived from the adrenal medulla, the lymphoid organs such as spleen that contain NK cells are heavily innervated by the sympathetic nerves, and the neural norepinephrine may also affect NK cells in these organs, as described by Benschop et al. (3).

Benschop et al. (1, 2) showed that epinephrine and norepinephrine cause detachment of NK cells from cultured endothelium and might induce recruitment of NK cells from the marginalizing pool to the circulation. Our present observations that norepinephrine downmodulates CD44 on NK cells and inhibits NK cell binding to hyaluronic acid appear to be relevant to the detachment of NK cells from endothelium. Thus it is likely that the catecholamine-induced mobilization of NK cells during exercise is primarily caused by the downmodulation of adhesion molecules on NK cells.

The downmodulation of adhesion molecules by catecholamines appears to be a unique feature of NK cells. The expression of CD44, CD62L, and CD18 on CD3^+ CD56^+ T cells did not change during and after exercise or by in vitro stimulation with norepinephrine (data not shown). This is consistent with the previous report that the change in T cell numbers is less than that of NK cells during exercise (8, 22). This appears to result from higher expression of β-adrenergic receptors on NK cells than on T cells (27).

Our present study suggests that the mobilization of NK cells during exercise is caused by downmodulation of NK cell surface adhesion molecules by catecholamines. In contrast to mobilization of NK cells during exercise, circulating NK cells rapidly decreased below the resting level after the exercise (Fig. 1). This decrease of NK cells might be associated with upregulation of CD44 and CD62L expression on NK cells, although the change was not statistically significant in the present study because of large individual differences. Further studies are needed to explain the decrease of circulating NK cells after exercise.

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**REFERENCES**


