N-acetylcysteine does not affect the lymphocyte proliferation and natural killer cell activity responses to exercise

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Nielsen, H. B., N. H. Secher, M. Kappel, and B. K. Pedersen. N-acetylcysteine does not affect the lymphocyte proliferation and natural killer cell activity responses to exercise. Am. J. Physiol. 275 (Regulatory Integrative Comp. Physiol. 44): R1227–R1231, 1998.—This study evaluated whether N-acetylcysteine (NAC) attenuates the reduced lymphocyte proliferation and natural killer (NK) cell activity responses to exercise in humans. Fourteen oarsmen were double-blind randomized to either NAC (6 g daily for 3 days) or placebo groups. During 6-min “all-out” ergometer rowing, the concentration of lymphocytes in the peripheral blood increased, with no significant difference between NAC and placebo as reflected in lymphocyte subsets: CD4+, CD8+, CD16+, and CD19+ cells. The phytohemagglutinin-stimulated lymphocyte proliferation decreased from 9,112 ± 2,865 to 5,851 ± 1,588 cpm (P < 0.05), but it was not affected by NAC. During exercise, the NK cell activity was increased from 17 ± 3 to 38 ± 4% and it decreased to 7 ± 1% below the resting value 2 h into recovery. Yet, when evaluated as lytic units per CD16+ cell, the NK cell activity decreased during and after exercise without a significant effect of NAC. We conclude that NAC does not attenuate the reduction in lymphocyte proliferation and NK cell activity associated with intense exercise.

METHODS

Fourteen male oarsmen (age 27 ± 1 yr, weight 80 ± 2 kg, height 189 ± 2 cm, VO2max 5.1 ± 0.2 l/min; mean ± SE) participated in the study after informed consent and approval by the Ethics Committee of Copenhagen (KF 02–132/93) and the Danish National Board of Health (5312–348–1993). No subject had any disease or injury 3 wk before the experiment, nor were the subjects taking any medication. They were not allowed to drink or eat after midnight on the day of the experiment, which began at 8:00 AM, and exercise within 24 h of the experiment was prohibited. At 9:00 AM a catheter was placed through an antecubital vein to the superior caval vein.

On 2 days separated by 3 wk, the subjects were randomized to either a N-acetylcysteine (each pill was 300 mg Astra, Copenhagen, Denmark) or a placebo (ASTRA) group in a double-blind crossover design. Three grams were administered with the morning and evening meals for 3 days before the experiment, and also 2 h before the exercise protocol began. Thus 20 pills were taken each day for a period of 3 days. This dose represented the highest tolerable dose necessary to maintain subject compliance despite the low bioavailability of N-acetylcysteine (4–10%; Ref. 11). No toxic side effects were reported, but the subjects reported that they felt “stomach gas” during one of the trials and this corresponded to the N-acetylcysteine period.

Exercise was performed on a rowing ergometer (Concept II, type C; Dreisacker, Morrisville, VT) connected to a computer (Concept II), which displayed the split time for every 500 m. All rowers warmed up at an individually determined pace for 10 min, recovered for 2 min, and then rowed “all-out” for 6 min. The exercise protocol was designed to simulate a 2,000-m competitive effort, and the subjects were familiar with this type of exercise. Heart rate was obtained by a “Sport Tester” PE 3000 (Polar Electro, Kempele, Finland).

Blood samples were drawn after 15 min of supine rest, during the last minute of exercise, and 1 and 2 h into the recovery. The subjects were offered soft drinks (noncaffeinated) and water ad libitum after exercise, but intake of coffee and tea was not allowed. No restrictions in food intake were made, but eating and drinking were not allowed within 30 min of blood sampling after exercise. Three milliliters of blood were drawn in sterile tubes with potassium-EDTA, and subsequent determination of the total lymphocyte count was made by a cell counter (Technicon, New York, NY). Twenty milliliters of blood for analysis of blood mononuclear cells (BMNC) were drawn in tubes with 100 μl of 20 U heparin.

The BMNC were isolated by gradient centrifugation (Lymphoprep; Nyegaard, Oslo, Norway) on Leucosep tubes (Greiner, Frickenhausen, Germany), washed three times in RPMI 1640 medium (Biological Industries, Kibbutz Beth Haemek, Israel) supplemented with 58.4 μg/ml L-glutamine (Sigma, St. Louis,
MO), 50 μg/ml gentamycin (ICN Biomedicals, Costa Mesa, CA), and 10% heat-inactivated natural human serum (NHS) to a final concentration of 10 × 10^6 cells/ml. Cells were distributed into cryotubes (Nunc, Roskilde, Denmark) with 1.5 ml in each tube and frozen in liquid nitrogen. Cells were then thawed in water at 37°C, and BMNC were resuspended in RPMI 1640 supplemented with 500 μg/ml streptomycin (NOVO, Copenhagen, Denmark), 500 U/ml penicillin (Leo, Ballerup, Denmark), and 58.4 μg/ml L-glutamine (Sigma). Cell viability was assessed via trypan blue technique.

Lymphocyte subpopulations were determined by monoclonal antibodies: FITC conjugated: Leu-11 (CD16^+), CD45RA^+ (Becton Dickinson, Oxnard, CA), Leu12 (CD19^+; Dako), LeuM3 (CD14^+; Becton Dickinson); PE-conjugated: Leu-19 (CD56^+; Becton Dickinson), CD45RO^+ (Dako); PerCP-conjugated: T3, (CD3^+), T4, (CD4^+), T8 (CD8^+; Dako, Glostrup, Denmark). The BMNC were washed twice in PBS supplemented with FCS to a solution of 3% FCS. Ten microliters of each antibody were added to 100 μl of BMNC. After incubation for 45 min at 5°C, the cells were washed twice in 3% FCS-PBS. Labeled cells were resuspended in 150 μl of 3% FCS-PBS and analyzed by flow cytometry with a fluorescence-activated cell sorter (FACStar, Becton Dickinson). The BMNC were incubated with the following combination of antibodies: CD3^+ alone, CD19^+ alone, CD4^+ with CD45RA^+, CD4^+ with CD45RO^+, CD8^+ with CD45RA^+, CD8^+ with CD45RO^+, CD16^+ with CD56^+, CD16^+ with CD8^+, and CD56^+ with CD8^+. The cell populations were located in a lymphocyte gate by forward and side scatter, and the cell percentage of each BMNC subset was multiplied by the total lymphocyte count representing the concentration of lymphocyte subpopulations in peripheral blood.

Lymphocyte proliferation was assayed as triplicate cell cultures (Nunc) in RPMI 1640 medium supplemented with 58.4 μg/ml L-glutamine (Sigma), 500 U/ml penicillin (Leo), 500 mg/ml streptomycin (NOVO), and 10% heat-inactivated NHS. Cells (160 μl of 0.58 × 10^6 cells/ml) were either unstimulated or stimulated for 72 h with the mitogen phytohemagglutinin (3 µg/ml; Difco Laboratories, Detroit, MI). The cultures were collected on glass fiber filters with a harvesting machine (Micromate 196 Harvester; Packard, Canberra, Australia) and the ^3H]thymidine incorporation was measured in a direct μ-counter (Matrix 96; Packard). For each triplicate, counts per minute were determined and presented as the average value.

The NK cell activity of BMNC was determined using K-562 target cells in a ^51Cr-release assay. Triplicates of 100 μl of BMNC effector cells (10 × 10^6, 5 × 10^6, 2.5 × 10^6, 1.25 × 10^6 cells/ml) and 100 μl of K-562 target cells (10 × 10^6 cells/ml) were incubated in microtiter plates for 4 h. Thus the effector-to-target cell ratios were 100:1, 50:1, 25:1, and 12.5:1. The plates were centrifuged for 6 min; 100 μl of the supernatant was transferred to new tubes and the released radioactivity was determined in counts per minute (Selektronic, Ringsted, Denmark). Spontaneous release was determined by incubation of 100 μl of target cells including 100 μl of 10% RPMI 1640 medium. Maximum release was determined by incubation of 100 μl target cells plus 100 μl 10% Triton X-100. Percentage ^51Cr release (NK cell activity) was determined as %lysis = [cpm_{sample} - cpm_{spontaneous}]/[cpm_{maximum} - cpm_{spontaneous}] × 100 and presented as the mean of triplicates. A lytic unit (LU) was defined as the number of effector cells required to lyse a specified percentage of target cells (4). LU was the number of effector cells required to lyse 20% of 10,000 target cells with results presented as the number of LU per 10^7 cells (4). LU, adjusted on a per-CD16^+ cell basis, was LU/(10^7 × (CD16^+/100)) (21, 22) as the CD16^+ cells exhibit significant target cell lysis (34).

Resting, exercise, and recovery values for both the placebo and N-acetylcysteine trials were analyzed using a Friedman analysis of variance. Pairwise differences were located by Wilcoxon signed rank sum test. Statistical significance was set at the 95% confidence limit (P < 0.05), and all data are presented as means ± SE.

Table 1. Concentration of blood mononuclear cells in trained humans at rest and in response to maximal exercise with either placebo or N-acetylcysteine

<table>
<thead>
<tr>
<th>Lympho</th>
<th>CD3^+</th>
<th>CD4^+</th>
<th>CD4^+/CD8^+</th>
<th>CD4^+/CD56^+</th>
<th>CD8^+</th>
<th>CD8^+/CD56^+</th>
<th>CD8^+/CD16^+</th>
<th>CD8^+/CD3^+</th>
<th>CD16^+</th>
<th>CD16^+/CD8^+</th>
<th>CD16^+/CD56^+</th>
<th>CD16^+/CD19^+</th>
<th>CD16^+/CD4^+</th>
<th>CD19^+</th>
<th>CD4^+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rest</td>
<td>1.5 ± 1.1</td>
<td>1.0 ± 0.1</td>
<td>0.7 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td>0.3 ± 0.0</td>
<td>0.1 ± 0.0</td>
<td>0.3 ± 0.0</td>
<td>0.3 ± 0.0</td>
<td>0.3 ± 0.0</td>
<td>0.2 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.5 ± 0.1</td>
<td>0.0 ± 0.0</td>
<td>0.2 ± 0.0</td>
</tr>
<tr>
<td>Exercise</td>
<td>4.0 ± 0.6*</td>
<td>1.9 ± 0.2*</td>
<td>0.7 ± 0.1*</td>
<td>0.6 ± 0.1*</td>
<td>0.3 ± 0.2*</td>
<td>0.2 ± 0.0*</td>
<td>0.2 ± 0.0*</td>
<td>0.2 ± 0.0*</td>
<td>0.2 ± 0.0*</td>
<td>0.2 ± 0.0*</td>
<td>0.2 ± 0.0*</td>
<td>0.0 ± 0.0*</td>
<td>0.1 ± 0.0*</td>
<td>0.0 ± 0.0*</td>
<td>0.1 ± 0.0*</td>
</tr>
<tr>
<td>1 h</td>
<td>0.9 ± 0.2*</td>
<td>7.0 ± 0.1*</td>
<td>5.0 ± 0.1*</td>
<td>2.0 ± 0.0*</td>
<td>2.0 ± 0.0*</td>
<td>1.0 ± 0.0*</td>
<td>1.0 ± 0.0*</td>
<td>1.0 ± 0.0*</td>
<td>1.0 ± 0.0*</td>
<td>1.0 ± 0.0*</td>
<td>1.0 ± 0.0*</td>
<td>0.8 ± 0.0*</td>
<td>0.8 ± 0.0*</td>
<td>0.8 ± 0.0*</td>
<td>0.8 ± 0.0*</td>
</tr>
<tr>
<td>2 h</td>
<td>1.0 ± 0.3*</td>
<td>0.7 ± 0.1*</td>
<td>0.5 ± 0.1*</td>
<td>0.2 ± 0.0*</td>
<td>0.2 ± 0.0*</td>
<td>0.1 ± 0.0*</td>
<td>0.1 ± 0.0*</td>
<td>0.1 ± 0.0*</td>
<td>0.1 ± 0.0*</td>
<td>0.1 ± 0.0*</td>
<td>0.1 ± 0.0*</td>
<td>0.1 ± 0.0*</td>
<td>0.1 ± 0.0*</td>
<td>0.1 ± 0.0*</td>
<td>0.1 ± 0.0*</td>
</tr>
</tbody>
</table>

Values are means ± SE in 10^7 cells/l. Lympho, concentration of lymphocytes. Other variables are the concentration of different lymphocyte subsets (see text). *Value different from rest, P < 0.05. There was no difference between placebo and N-acetylcysteine variables.
RESULTS

During maximal exercise with both N-acetylcysteine and placebo, the oarsmen completed 1,804 ± 24 m (356 ± 12 W) and heart rate was elevated to 187 ± 2 beats/min. The percentages of CD3+, CD4+, CD4-/CD45RO+, and CD4+/CD45RA+ decreased during exercise. The percentages of CD16+, CD56+, CD16+/CD56+, CD16+/CD8+, CD56+/CD8+, and CD19+ increased and the CD8+ cells remained unchanged (data not shown). Following exercise, only the percentages of CD8+ and CD16+/CD56+ cells had decreased below resting levels, whereas the percentage of CD14+ cells remained stable. The lymphocyte count and the concentration of all lymphocyte subsets increased during exercise and decreased below the level at rest 2 h into the recovery period (Table 1). N-acetylcysteine did not influence the concentrations and percentage distribution of lymphocyte subsets.

The unstimulated lymphocyte proliferation with 2-mercaptoethanol was not elevated but it became enhanced with phytohemagglutinin stimulation (Table 2). During exercise, the unstimulated lymphocyte proliferation decreased and the phytohemagglutinin-stimulated proliferation decreased below that at rest with no significant effect of N-acetylcysteine. During the recovery period after exercise, lymphocyte proliferation of unstimulated cells decreased, whereas the response of phytohemagglutinin-stimulated cells was similar to resting levels. The added 2-mercaptoethanol failed to increase the mitogen-stimulated proliferation. However, a low concentration of 2-mercaptoethanol reduced proliferation at rest, whereas only a high concentration reduced proliferation during exercise. Inhibition of glutathione synthesis by BSO reduced proliferation similarly at rest (2,697 ± 216 vs. 2,418 ± 228 cpm, P = 0.07) and during exercise (2,096 ± 226 vs. 1,918 ± 212 cpm, P = 0.07) in placebo and N-acetylcysteine groups.

The NK cell activity increased during exercise, but it was reduced to a level below that during rest both 1 and 2 h into the recovery period (Fig. 1). The LU increased during exercise, but expressed on a per CD16+ cell basis, neither exercise nor N-acetylcysteine had a significant influence. However, when only the effect of exercise was evaluated, LU per CD16+ cell was reduced both during and after exercise in both treatments.

DISCUSSION

In relation to maximal exercise, the impaired NK cell activity and mitogen-stimulated lymphocyte proliferation were not abolished by N-acetylcysteine. Furthermore, N-acetylcysteine did not influence the concentra-

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**Table 2. Lymphocyte proliferation with either placebo or N-acetylcysteine in response to exercise either unstimulated or stimulated with PHA and three concentrations of 2-mercaptoethanol**

<table>
<thead>
<tr>
<th></th>
<th>Rest M0</th>
<th>Exercise M1</th>
<th>Recovery 1h M2</th>
<th>Recovery 2h M3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>45 ± 8</td>
<td>50 ± 8</td>
<td>52 ± 6*</td>
<td>50 ± 8</td>
</tr>
<tr>
<td></td>
<td>49 ± 7</td>
<td>46 ± 7</td>
<td>59 ± 8*</td>
<td>47 ± 7</td>
</tr>
<tr>
<td></td>
<td>10,374</td>
<td>7,942†</td>
<td>8,370</td>
<td>9,566</td>
</tr>
<tr>
<td></td>
<td>± 2,985</td>
<td>± 2,007</td>
<td>± 2,542</td>
<td>± 1,588</td>
</tr>
<tr>
<td>N-acetylcysteine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>58 ± 9</td>
<td>60 ± 7</td>
<td>61 ± 10</td>
<td>47 ± 6</td>
</tr>
<tr>
<td></td>
<td>50 ± 8</td>
<td>52 ± 7</td>
<td>62 ± 10</td>
<td>42 ± 7</td>
</tr>
<tr>
<td></td>
<td>10,639</td>
<td>7,418</td>
<td>8,530</td>
<td>9,564</td>
</tr>
<tr>
<td></td>
<td>± 3,485</td>
<td>± 2,594</td>
<td>± 2,959</td>
<td>± 1,719</td>
</tr>
<tr>
<td>PHA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>60 ± 7</td>
<td>76 ± 12</td>
<td>68 ± 12</td>
<td>50 ± 8</td>
</tr>
<tr>
<td></td>
<td>54 ± 7</td>
<td>52 ± 7</td>
<td>62 ± 10</td>
<td>42 ± 7</td>
</tr>
<tr>
<td></td>
<td>17,024</td>
<td>9,054</td>
<td>10,379</td>
<td>11,277</td>
</tr>
<tr>
<td></td>
<td>± 4,762</td>
<td>± 3,394</td>
<td>± 4,296</td>
<td>± 2,369</td>
</tr>
</tbody>
</table>

Values are means ± SE in counts per minute radioactivity of [3H]thymidine. M0, without mercaptoethanol; M1, mercaptoethanol 5 × 10−3 µg/l; M2, mercaptoethanol 5 × 10−4 µg/l; M3, mercaptoethanol 5 × 10−5 µg/l. *Different from M0; †Different from rest, P < 0.05. There were no significant differences between placebo and N-acetylcysteine variables.
Exercise-induced changes in lymphocyte proliferation and NK cell activity are well established, but the mechanisms involved in these are not clear (10, 26). Treatment with N-acetylcysteine and the induction of cellular cysteine uptake by 2-mercaptoethanol did not increase lymphocyte proliferation, and BSO failed to reduce lymphocyte proliferation to the exercise level. These observations suggest that cellular cysteine and also glutathione are not reduced to a level critical for lymphocyte function. In addition, reactive O₂ species may not contribute to reduce proliferation as N-acetylcysteine is antioxidative in response to exercise (13, 30). Other factors could be of importance. Phytoglobin stimulates predominately T lymphocytes (18), and, with a reduced percentage of CD4⁺ cells (33), the lymphocyte proliferative response will be reduced. Thus, when the lymphocyte proliferation was calculated on a per percentage of CD4⁺ cell basis, it is not changed by intense exercise.

The NK cell activity is known to increase during exercise (10, 19, 20) in proportion to the increase in CD16⁺ cells (26), although the two responses are not of the same magnitude (20). After exercise, the NK cell activity decreased, whereas the percentage of CD16⁺ was at the resting level, suggesting an attenuated activity in each NK cell. Evaluated as LU on a per-CD16⁺ cell basis, the NK cell activity was reduced, indicating that each NK cell contributed less to target cell lysis both during and after exercise. Such a response may correspond to an effect of reactive O₂ species (24) and a peroxidation process of membrane lipids (14). Furthermore, N-acetylcysteine increases the NK cell lysis of the NK cell-resistant U-937 target cells in vitro (16), but, in the present study, the NK cell lysis of K-562 target cells was not affected. Either N-acetylcysteine was unable to inhibit reactive O₂ species in response to exercise or the oxidative stress was not at a level where the NK cell function became affected in vivo.

A reduced NK cell activity could reflect redistribution of lymphocytes. The NK cells express a high number of β-adrenergic receptors (15), and intense sympathetic activation, especially that found during rowing (12), may affect NK cells possessing a low lytic capacity. These cells might also remain in peripheral blood, and the NK cell activity will be reduced. Both the intensity and duration and the muscle mass involved during exercise appear to be of importance. The LU, calculated on a per NK cell basis, is reduced with resistance training to exhaustion (22), whereas it is unaffected by 16 min of incremental cycling to a maximal intensity (21).

N-acetylcysteine was administered orally at a high, tolerable dose to minimize the effect of deacetylation in the gut. An oral dose of [³⁵S]acetylcysteine (100 mg) increases plasma radioactivity for 24 h (28), and repeated doses of N-acetylcysteine (200 mg) maintain a high plasma concentration for 8 h (6). After a single oral dose of N-acetylcysteine, free and total N-acetylcysteine disappear from the circulation with a half-life of ~1 h (5), and cysteine is the major metabolite (31). In relation to exercise, an oral dose of 800 mg administered daily for 2 days reduces the production of reactive O₂ species by neutrophil granulocytes (13). Furthermore, N-acetylcysteine attenuates an increase of oxidized glutathione in plasma (30).

We propose that intracellular cysteine is not reduced to a level critical for glutathione synthesis during exercise. An alternative hypothesis is that N-acetylcysteine was not able to increase a low level of intracellular glutathione, although the level of plasma cysteine becomes elevated (35). The concentration of plasma cysteine was also elevated with a lower dose of N-acetylcysteine (3), similar to that used by Huuponen et al. (13) and Sen et al. (30). An effect of N-acetylcysteine on lymphocytes may be abolished when N-acetylcysteine is administered in a high oral dose as an elevated level of cysteine (glutathione) exhibits negative feedback on the γ-glutamylcysteine synthetase (27).

In a high, tolerable, oral dose, N-acetylcysteine does not prevent the reduction in lymphocyte proliferation and NK cell activity in response to exercise, suggesting that cysteine availability does not play a significant role. During exercise, a determination of the level of cysteine and glutathione in the lymphocytes might be a productive area for future research.

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