β-Endorphin and natural killer cell cytolytic activity during prolonged exercise. Is there a connection?

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This study was designed to test whether a single 50-mg dose of the opioid antagonist naltrexone hydrochloride, ingested 60 min before 2 h of moderate-intensity exercise (i.e., 65% peak O2 consumption), influenced the exercise-induced augmentation of peripheral blood natural killer cell cytolytic activity (NKCA). Ten healthy male subjects were tested on four occasions separated by intervals of at least 14 days. A rested-state control trial was followed by three double-blind exercise trials [placebo (P), naltrexone (N), and indomethacin (I)] arranged according to a random block design. The indomethacin exercise trial was discussed elsewhere (S. G. Rhind, G. A. Gannon, P. N. Shek, and R. J. Shephard, Med. Sci. Sports Exerc. 30: S20, 1998). For both the P and N trials, plasma levels of β-endorphin were increased (P < 0.05) at 90 and 120 min of exercise but returned to resting levels 2 h postexercise. CD3 and CD65 NK cell counts were significantly (P < 0.05) elevated at each sampling time. Changes in NK cell counts or NKCA between P and N trials at any time point during the two trials. Changes in NKCA reflected mainly changes in NK cell count (r = 0.72; P < 0.001). The results do not support the hypothesis that the enhancement of NKCA during prolonged submaximal aerobic exercise is mediated by β-endorphin.

naltrexone; natural immunity; cell adhesion; growth hormone; cortisol

PHYSICAL ACTIVITY of sufficient intensity and duration stimulates the release of β-endorphin from the anterior pituitary gland, increasing blood levels of this hormone (18). Although β-endorphin is known to have immunomodulatory properties (38), the biological significance of the increased plasma concentrations that are seen during exercise remains unclear (19, 25). In vitro, β-endorphin acutely enhances the cytolytic activity of peripheral blood natural killer (NK) cells through an opioid receptor-mediated, naloxone-reversible pathway (32, 34), suggesting that NK cells carry specific receptors for β-endorphin (5, 41). The dose-response curve apparently has an inverted-U shape, although there is little agreement on the minimum concentration required to induce a positive effect; enhancement of NK cell-mediated cytolytic activity (NKCA) has been demonstrated at concentrations ranging widely from 10^{-14} to 10^{-6} mol/l (6, 12, 28).

Because physical activity exerts a well-demonstrated stimulatory influence on the NKCA of peripheral blood (14), the physiological plasma concentrations of β-endorphin (i.e., 10^{-12} mol/l) induced by physical exercise could conceivably explain the acute effects of physical exercise on NKCA. In support of this, Fiatarone et al. (11) administered in a blind protocol either normal saline or the opioid antagonist naloxone (100 µg/kg) to eight healthy young women who then performed a maximal incremental cycle ergometer test. After naloxone administration, the rise in NKCA was no longer statistically significant, although the increase in peripheral blood NK cell count (identified with CD16 or CD56 surface markers) was similar to that seen in the placebo trial. In contrast, Kappel et al. (27) argued that the exercise-induced increase in NKCA could be explained entirely by a concomitant increase in peripheral blood NK cell number, secondary to sympathetic activation and the peripheral release of epinephrine, although others have argued that not all of the effect of exercise on NKCA can be attributed to changes in NK cell number (31, 52). In support of the hypothesis of Kappel et al. (27), a similar cytolytic activity per NK cell (per NKCA) was seen before, during, and subsequent to exercise (4). Further evidence against a role for β-endorphin was provided by a study of seven healthy young men in which lumbar epidural anesthesia was used to block afferent nerve impulses from exercised skeletal muscles (30), thus preventing any increase in β-endorphin during 20 min of recumbent cycle ergometry (60% peak O2 consumption [VO2peak]) under hypoxic conditions. The anticipated exercise-induced increase in NKCA and NK cell concentration was unaffected by the sensory nerve blockade.

Because cell adhesion represents the first step of effector-target interaction, it is logical to reason that any stimulatory effect induced by β-endorphin may be mediated by an increased expression of specific cell surface adhesion molecules. Lymphocyte function-associated antigen 1 (LFA-1; CD11a/CD18) and LFA-2 (CD2) are two important cell adhesion molecules; they...
are expressed by peripheral blood NK cells and function as "accessory" molecules during cell-cell communication and activation (44, 46). In particular, these molecules and their respective target cell ligands (intracellular adhesion molecule 1 and LFA-3) play a major role during the recognition, conjugation, and cytolyis of K562 target cells by peripheral blood NK cells (47, 53).

The present study used the nonselective opioid receptor antagonist naltrexone hydrochloride to investigate further the possible influence of β-endorphin on NKCA during and after an acute bout of prolonged physical activity. Therapeutic doses were administered according to a randomized, double-blind, placebo-controlled protocol. We chose a 2-h bout of moderate aerobic activity (i.e., 60–70% VO₂peak), envisioning that the first hour of such exercise would increase the NK cell count but not plasma β-endorphin and that the second hour would increase plasma β-endorphin (48) without further increments in NK cell count. The effects of naltrexone hydrochloride on the mean surface density of CD11a (LFA-1α) and CD2 cell surface adhesion molecule expression were examined, and we controlled for secondary effects of naltrexone hydrochloride on growth hormone (GH) and cortisol (10, 36), which have a demonstrated influence on lymphocyte trafficking and NKCA (39, 40).

METHODS

Subjects. Ten recreationally active (VO₂peak 44.0 ± 3.5 ml·kg⁻¹·min⁻¹) male nonsmokers [age 26.3 ± 5.4 (SD) yr, mass 79.3 ± 10.3 kg, height 1.78 ± 0.07 m] volunteered to participate in this study under conditions approved by the University of Toronto and the Defence and Civil Institute of Environmental Medicine Human Experimentation Committees. Participation was contingent on a detailed medical examination and approval by a physician. A history of allergies or evidence of acute or chronic infection were criteria for exclusion from the study.

Physical assessment. After clinical examination and medical clearance, but at least 1 wk before the control trial, VO₂peak and peak heart rate (HRpeak) were determined on a mechanically braked cycle ergometer (Monark Ergomedic 818E; Stockholm, Sweden). Subjects performed an incremental exercise test at a pedal cadence of 70 rpm (initial loading of 60 W, with 25-W/min increments). Volitional exhaustion was reached in 8–12 min. Expired gases, collected breath by breath, were analyzed for respiratory minute volume and oxygen consumption using a metabolic measurement cart (SensorMedics 2900C, Yorba Linda, CA). Heart rates were recorded at 5-s intervals using a Polar Vantage XL heart rate monitor (Polar USA). The work rate estimated to elicit 65% VO₂peak was determined for each subject from a plot of work rate versus oxygen consumption.

Control and experimental trials. Within 2 wk of the physical assessment, subjects performed a control trial followed by three randomized, counterbalanced exercise trials at intervals of at least 2 wk. On each test day, subjects reported to the laboratory at 0700 to 0730, having fasted overnight and abstained from strenuous physical activity for 36 h. Subjects were immediately instrumented with a heart rate monitor and a 21-gauge intravenous catheter (Insize, BD Vascular Access, Sandy, UT). To standardize metabolic conditions, each subject consumed 1.1 MJ (250 cal) of a clinical dietary product (Ensure Plus; 9.4 g protein, 38 g carbohydrate, and 6.7 g fat) immediately after collection of the initial (T₀) blood sample.

The nonexercise control trial was conducted in the same laboratory environment as the exercise trials, serving to familiarize subjects with personnel, protocol, and equipment. Beginning between 0730 and 0800, after the subjects had ~30 min of seated rest, we collected serial blood samples according to the following schedule: (in hours) time 0 (T₀), T₁, T₂, T₄, and T₂₄.

The exercise trials involved administration of either placebo or naltrexone hydrochloride before a 2-h bout of cycle ergometry at a pedal cadence of 60 rpm and an intensity adjusted to demand 65% of the individual’s VO₂peak. Oxygen consumption (SensorMedics 2900C, Yorba Linda, CA) and heart rate were recorded using a Polar Vantage XL for 15 min during exercise, and the work load was adjusted as necessary to maintain the required intensity of effort. Participants were encouraged to consume 1.0–1.5 liters of water during each trial to minimize hemococoncentration. Venous blood samples of 45 ml were collected in sterile glass Vacutainers (Becton-Dickinson, Franklin Lakes, NJ) containing the necessary preservatives and anticoagulants at T₀, T₅, T₁, T₁₅, T₄, and T₂₄ relative to the initiation of exercise.

Drug administration. Identical gelatin capsules containing lactose placebo (180 mg; Novopharm, Scarborough, Ontario, Canada) or naltrexone hydrochloride (50 mg; Trexan, Du Pont Merck Pharmaceuticals, Wilmington, DE) were presented according to a double-blind, counterbalanced protocol immediately after collection of the initial blood sample and 60 min before exercise. Compliance was controlled by observation of drug and/or placebo intake on scheduled test days.

Hematological analyses. Determinations of total leukocyte counts, three-cell differential counts (granulocytes, monocytes, and lymphocytes), Hb, and hematocrit were performed on tripotassium ethylenediamine tetra-acetate-treated blood using an automated Coulter®T hematolyzer analyzer (Coulter Electronics, Hialeah, FL). All blood cell counts were corrected to resting (T₀) blood volumes using the observed Hb and hematocrit values and applying the formulas of Dill and Costill (9).

MAbs. The following mouse anti-human monoclonal antibodies (MAbs) were used in this study: FITC anti-CD11a MAb B-B15 (IgG₁) and FITC anti-CD2 MAb LT2 (IgG₂a), purchased from Serotec Canada (Mississauga, Ontario, Canada); and FITC anti-CD3 MAb SK7 (IgG₁), phycoerythrin (PE) anti-CD16 MAb B73.1 (IgG₁), and PE anti-CD56 MAb MY31 (IgG₁), purchased from Becton-Dickinson (Mississauga, Ontario, Canada).

NK cell immunophenotyping. NK cells (CD3⁻ CD16⁺ CD56⁺) were enumerated by dual-parameter immunophenotyping, using combinations of FITC- and PE-conjugated MAbs. All samples were analyzed on the day when they were collected. Briefly, 100 µl EDTA-whole blood was incubated with saturating amounts of FITC anti-CD3, PE anti-CD16, and PE anti-CD56 MAbs as previously described (13). Whole blood samples with leukocyte counts >9.8 × 10⁹ cells/l were diluted with 1× isosonic PBS containing 0.1% sodium azide. Stained-
cell suspensions were enumerated on a FACScan flow cytometer equipped with an air-cooled argon ion laser emitting 15 mW at 488 nm using standard operating methods (Becton-Dickinson Immunocytometry Systems (BDIS), San Jose, CA). Daily calibration was performed using a mixture of monosized FITC- and PE-conjugated and unconjugated latex particles (CaliBRITE beads, BDIS) in conjunction with AutoCOMP software (BDIS). Forward-scatter and side-scatter gains, forward-scatter threshold, and fluorescence compensation levels were optimized using isotype-negative controls (anti-IgG1-FITC/anti-IgG1-PE) and anti-CD4-FITC/anti-CD8-PE double-stained whole blood samples. Electronic compensation was adjusted to eliminate spectral overlap between fluorescence channels. Digitized data were acquired and analyzed using CellQuest software (BDIS).

Cell adhesion molecule surface density. Peripheral blood mononuclear cell (PBMC) suspensions were stained with saturating concentrations of PE anti-CD56 MAb and a second FITC-labeled MAb specific for either CD11a or CD2 (13). Stained-cell suspensions were analyzed for fluorescence on a multiparameter FACScan flow cytometer (BDIS). Acquisition list mode data were analyzed with CELLQuest software (BDIS) for mean fluorescence intensity (MFI) of CD11a and CD2 on CD56-positive lymphocytes (i.e., total cellular fluorescence of CD56-positive lymphocytes averaged over the number of positive events; MFI). For each analysis, 5,000 events positive for CD56 were acquired. The MFI served as an indicator of mean surface density of a given adhesion molecule.

Isolation of PBMCs. Fresh PBMCs were isolated from heparinized blood samples (143 USP units/10 ml of blood) by 30 min of density-gradient centrifugation (400 g, 20°C) over Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). The mononuclear cell band was carefully harvested, washed, and reconstituted to a concentration of 2.0 × 10^7 cells/ml in 10% FCS-RPMI 1640.

K562 tumor cell culture. The NK-sensitive K562 tumor line (American Type Culture Collection, Rockville, MA) was used to provide target cells in cytolytic assays. The cell line was maintained as a continuous suspension in RPMI 1640 culture medium containing 10% FCS, 1% (wt/vol) penicillin-streptomycin, 20 mM HEPES (pH 7.3), and 2 mM L-glutamine (Gibco Life Technologies, Burlington, Ontario, Canada). A humidified, water-jacketed incubator (Revco Ultima; VWR Scientific, Toronto, Ontario, Canada) provided a 37°C atmosphere of 5% CO_2 to maintain a pH of 7.2–7.3. To ensure that cells were in the logarithmic phase of growth, cultures were split into 3–5 × 10^5 cells/ml on a 24-h schedule 3 days before the experiment. Cell viability was assessed by trypan blue exclusion and was typically >90%.

Tumor cell labeling. K562 tumor cells were first washed two times in 10 ml RPMI 1640 medium without FCS and centrifuged for 5 min (400 g, 20°C); they were then resuspended at a concentration of 1 × 10^7 cells/ml. K562 tumor cells were labeled with PKH26 (Sigma, St. Louis, MO), a stable lipophilic membrane dye with an emission peak at 567 nm. To label cells, we rapidly added 0.5 ml of the target cell suspension to 0.5 ml of PKH26 (4 µM) in a 12 × 75 mm polystyrene culture tube. After incubation at 25°C for 2–5 min, the reaction was stopped by the addition of 1 ml of 100% FCS for 1 min. After centrifugation (400 g, 25°C) for 5 min, cells were washed (3 times) in 10 ml of supplemented RPMI 1640 medium and resuspended to a final concentration of 2 × 10^6 cells/ml for the cytolytic assay.

NKCA. The total cytolytic activity of peripheral blood NK cells was assessed by an in vitro flow cytometric assay (22). To expedite data acquisition, we modified this method by using a higher target cell concentration (43). With this method, the plasma membrane integrity of PKH26-labeled K562 tumor cells, after 4 h of incubation with PBMC (i.e., NK cells), is determined with flow cytometry using the DNA-intercalating dye propidium iodide (PI).

Briefly, 100 µl of freshly isolated PBMC (effectors, at 1 × 10^7 cells/ml) were gently mixed with 100 µl of PKH26-labeled K562 tumor cells (targets, at 2 × 10^5 cells/ml) and 25 µl (1 µg/ml) of PI solution (Sigma) at an effector-target ratio of 50:1. Cell mixtures were centrifuged for 5 min (20°C, 50 g) to promote optimal effector-to-target cell conjugation and incubated for 4 h (37°C, 5% CO_2). The assay was stopped by addition of cold cell wash to the cultures. Samples were placed on ice until same-day analysis.

Samples were analyzed in triplicate, using a FACScan flow cytometer and CellQuest software (BDIS). PKH26 target cells were defined and live gated via a histogram of FL2 fluorescence. A minimum of 5,000 PKH26+ target cell events (corresponding to ~200,000 list mode events) were acquired per sample. Dead K562 cells were differentiated from live K562 cells based on the FL3 fluorescence of PI. Spontaneous target cell death was determined by incubating 100 µl of PKH26-labeled K562 tumor cells with 25 µl of PI in the absence of effector cells. Percent specific lysis was calculated by subtracting the mean percentage of spontaneously dead target cells from the percentage of target cells killed in the test sample. The corresponding absolute number of dead target cells was calculated by multiplying the percent lysis by the total number of target cells used in a given assay. The intra-assay coefficient of variation was consistently 4% among triplicate samples, and the between-assay coefficients of variation for the same subject were typically 5%.

Neuroendocrine analyses. Total plasma β-endorphin concentrations were determined in duplicate, using an affinity gel extraction and RIA (Incstar, Stillwater, MN). Total plasma concentrations of cortisol (ICN Biomedicals, Costa Mesa, CA) and GH (Allegro Nichols Institute, San Juan Capistrano, CA) were determined in duplicate using a competitive solid-phase RIA technique. Plasma hormone concentrations were adjusted for estimated changes in plasma volume using the formulas of Dill and Costill (9).

Plasma naltrexone and 6-naltrexol analyses. Plasma samples (0.5 ml) were analyzed by gas chromatography (GC) and mass spectrometry (MS), using negative ion chemical ionization and a selective ion-monitoring mode. The method was adapted from Monti et al. (35), with methane-5% ammonia as the reagent gas and deuterium-labeled naltrexone and naltrexol as internal standards. Unknown plasma samples and plasma spiked with standards were extracted with toluene, dried at 40°C under a stream of nitrogen, and treated with pentafluoropropionic anhydride for 45 min at 80°C. Excess pentafluoropropionic anhydride was removed by dry-
significant increments at the exercise (Pr < 0.001), with a condition × time interaction. RM ANOVA was used to determine the main effects of condition (control vs. placebo vs. naltrexone), time (T0, T0.5, T1, T1.5, T2, T4, and T24), and condition × time interactions. Because values for a given measure are highly correlated across time, the Greenhouse-Geisser correction was applied to reduce the risk of a type I error. When a significant F ratio was demonstrated, differences among treatment means were determined by a Newman-Keuls post hoc analysis.

RESULTS

Physiological response to acute exercise. Mean oxygen costs of the exercise conditions (i.e., placebo and naltrexone) were maintained at −65% VO2peak as demonstrated by condition (F1,9 = 0.10, Pr = 0.76), time (F3,27 = 0.17, Pr = 0.91), and condition × time interaction (F3,27 = 0.67, Pr = 0.58) statistics (2 × 4 RM ANOVA). Absolute power outputs were 127 ± 14 and 125 ± 12 W for placebo and naltrexone, respectively, eliciting 82.0 ± 4.5 and 78.6 ± 4.2% of HRpeak.

Plasma naltrexone and 6β-naltrexol. Plasma naltrexone concentrations reached 9.7 ± 1.7 ng/ml within the first 30 min of exercise (i.e., 90 min postingestion), and values were still within 2.0 ± 0.2 ng/ml 2 h postexercise (Fig. 1A). The plasma concentration of the metabolite 6β-naltrexol peaked at 60 min of exercise (81.5 ± 7.6 ng/ml), falling to 11.1 ± 1.3 ng/ml by T24 (Fig. 1B). A plasma naltrexone concentration of 2 ng/ml provides effective opioid blockade (54).

Monocyte and NK cell counts. Initial (T0) circulating monocyte (data not shown) and CD3–CD16–CD56– NK cell (Fig. 2A) counts fell within normal resting ranges and did not differ significantly between conditions. In the control condition, no significant differences were found between T0 and T24 for monocyte counts. However, NK cell counts demonstrated a significant main effect of time (F2,321.1 = 3.29, Pr < 0.05); counts at T24 (0.23 ± 0.03 × 109 cells/l) were significantly greater (Pr < 0.05) than counts at T0 (0.17 ± 0.02 × 109 cells/l).

Over the three conditions, monocyte counts (data not shown) showed significant main effects of condition (F1,8,16.4 = 4.32, Pr < 0.05) and time (F2,421.3 = 21.84, Pr < 0.001), with a condition × time interaction (F4,0.36.2 = 5.73, Pr < 0.001). Relative to the corresponding control data, the moderately intensive placebo cycling protocol elevated the total circulating monocyte count by 104% (Pr < 0.05) at 30 min of exercise (T0.5), with further significant increments at 2 h of exercise (T2). By 2 h postexercise, the number of circulating monocytes had returned to control levels. The exercise-induced mobilization of monocytes was similar under both placebo and naltrexone conditions.

Significant main effects of condition (F1,5,13.6 = 42.92, Pr < 0.001) and time (F1,8,15.8 = 40.44, Pr < 0.001) and a condition × time interaction (F3,2,28.9 = 28.85, Pr < 0.001) were found for changes in circulating CD3–CD16–CD56– NK cell counts over the three conditions (Fig. 2A). During the placebo exercise condition, CD3–CD16–CD56– NK cell counts had increased by 270% by 30 min of exercise (T0.5), accounting for −20% of the total circulating lymphocyte pool. Despite the steadystate nature of the exercise protocol, counts continued to increase to 330% of resting control values at 2 h of exercise (T2). The NK lymphocytosis had abated by 2 h postexercise (T4), with counts dropping to 75% of the T4 control value. Normal resting values were achieved by 24 h postbaseline (T24). There were no statistically significant differences between the placebo and naltrexone conditions.

In vitro NKCA. The total cytolytic activity of NK cells against K562 target cells, measured as percent lysis (Fig. 2B), showed a significant main effect of time (F2,6,21.2 = 81.86, Pr < 0.001) and a significant interaction effect (F3,0.272 = 16.33, Pr < 0.001). Under placebo conditions, NKCA increased from 26.3 ± 3.4% lysis (T0) to 49.4 ± 6.0% lysis (T2) during exercise (85% increase, Pr < 0.01) but fell 28% below resting control values at T4 2 h postexercise (Pr < 0.01). A very similar trend was found in the naltrexone condition; post hoc analysis suggested that percent lysis values were not significantly different between conditions at any time point. However, 24-h postbaseline placebo data showed a
**β-ENDORPHIN, NK CELL CYTOLYTIC ACTIVITY, AND EXERCISE**

There was a significant (P < 0.05) relative to corresponding control values, whereas the naltrexone data did not. Measures of NKCA at baseline (T₀) showed a nonsignificant trend to a difference (P > 0.05) between placebo and naltrexone conditions (Fig. 2B). We thus decided to express the percent lysis data for each condition as a change relative to their corresponding baseline (T₀) values (Fig. 2C). This made the trend to differences between placebo and naltrexone conditions more apparent. Relative to corresponding baseline (T₀) values, there was a significant (P < 0.01) 33% drop in percent lysis at 2 h postexercise (T₄) during the naltrexone trial, whereas the 20% drop at 2 h postexercise during the placebo condition was not significant. Taken together, these results suggest that naltrexone administration may have reduced the natural cytotoxic capacity of the whole blood compartment early in recovery from prolonged aerobic exercise.

Per NKCA. The CD3⁺CD16⁻CD56⁺ NK cells accounted for an increasing proportion of the total circulating lymphocyte pool during exercise but returned approximately to baseline levels at 2 and 24 h postexercise (Fig. 2A). Exercise also changed the relative concentrations of circulating monocytes (data not shown), leading to significant changes in the composition of the 1.0 × 10⁶ mononuclear cells (i.e., PBMC) incubated at a 50:1 ratio with K562 cells. Thus, to determine the exact effector-to-target ratio (i.e., CD3⁺CD16⁻CD56⁺ NK cell: K562 cell) at each time point, adjustments were made to account for the exercise-induced changes in the proportion of NK cells and monocytes. The percentage of monocytes in 1.0 × 10⁶ PBMCs showed only minor shifts (interaction effect; F₃.₇₂₃₇ = 1.54, P = 0.21); however, at 2 h postexercise, both placebo and naltrexone values exceeded the corresponding control values (16.2 and 16.8%, respectively, vs. 12.5%). The calculated NK-K562 ratio changed from ~4:1 at baseline (T₀), over 9:1 during exercise (P < 0.01), and between 4 and 5:1 postexercise (T₄ and T₂₄) for both placebo and naltrexone conditions.

When NKCA was expressed on a per-CD3⁺CD16⁺ CD56⁺ NK cell basis (i.e., per NKCA), there was no statistically significant interaction effect of time versus condition (Fig. 2D). For the placebo condition, values of per NKCA showed a decreasing trend from baseline (T₀) value of 7.3 × 10⁻² dead K562 cells to below 6.0 × 10⁻² dead K562 cells during exercise, while increasing to ~6.3 × 10⁻² dead K562 cells at T₄ and T₂₄. No differences were found between the placebo and naltrexone conditions.

Because per NKCA did not change significantly during either placebo or naltrexone conditions, it appears that much of the change in the natural cytotoxic capacity of whole blood (i.e., difference between T₀ and T₂₄) can be accounted for by changes in the concentration of circulating NK cells (Pearson product moment correlation; r = 0.72, P < 0.001). To evaluate the relationship between NKCA and NK cell count, we fitted a linear regression relating the number of CD3⁺CD16⁺CD56⁺ NK cells to the number of dead K562 cells for each subject at each time point (Fig. 3); this demonstrated significant correlations for both pla-
cebo and naltrexone (P < 0.01), although the goodness of fit was slightly tighter for placebo (r = 0.818) than for naltrexone (r = 0.695).

Neuroendocrine response. Resting (T0) plasma β-endorphin levels (3.7 ± 0.7 pmol/l, averaged across conditions) were in the normal range and did not differ significantly between placebo and naltrexone conditions (Fig. 4A). In the placebo condition, β-endorphin increased by 235% (P < 0.05) and 300% (P < 0.05) at 90 (T1.5) and 120 min (T2) of exercise, respectively, relative to the corresponding baseline (T0) value (1-way RM ANOVA; F1,6,14.8 = 7.51, P < 0.01). A significant main effect of condition (F1,9 = 9.18, P < 0.01) indicated that, compared with placebo, β-endorphin concentrations were higher throughout the naltrexone condition. In the naltrexone condition, values were 250 and 400% of baseline (T0) at T1.5 and T2, respectively.

Resting values for total plasma GH (Fig. 4B) were within the expected range of 1–5 µg/dl. Exercise induced a significant increase (P < 0.01) in total GH concentrations, but values returned to the nonexercise control value 2 h postexercise (T4). There were no significant differences between placebo and naltrexone conditions.

Total plasma cortisol concentrations (Fig. 4C) showed significant main effects of condition (F1,8,12,2,8 = 4.34, P < 0.05) and time (F2,2,2,2,0 = 3.15, P < 0.05), with a significant interaction effect (F6,8,47,2,6 = 2.29, P < 0.05). The mean resting (T0) values fell within the expected range of 5–20 µg/dl, and subjects displayed the expected circadian changes (F2,2,12,3,1 = 4.69; P < 0.05); values decreased significantly (P < 0.05) from 11.5 ± 1.8 µg/dl at 0800 (T0) to 7.0 ± 1.3 µg/dl at 1200 (T4). Exercise values at T2 through T1.5 were not significantly different from the corresponding control values. However, cortisol concentrations at T2 were ~100% greater (P < 0.01) than the corresponding control values for both placebo and naltrexone conditions. There were no significant differences between placebo and naltrexone conditions.

Adhesion activation molecules. The mean surface density of CD11a (i.e., LFA-1α) and CD2 (LFA-2) on CD56+ lymphocytes was analyzed at each time point for all three experimental conditions (Fig. 5). Significant interaction effects were found for relative changes in CD11a surface densities (F2,8,25,0 = 3.79, P < 0.05) and CD2 surface densities (F2,5,22,1 = 3.28, P < 0.05).

In the placebo condition, the mean CD11a surface density of circulating CD56+ lymphocytes had increased (P < 0.05) by ~25 MFI units at 30 min of exercise (Fig. 5A). This level of expression was maintained until 2 h of exercise (T4). Similar changes were found during the naltrexone condition. Two hours postexercise (T4), the mean surface density had dropped to ~9 and 14 MFI units below the resting control for placebo and naltrexone conditions, respectively (P > 0.05). The only significant difference between the two
conditions was found at 24 h postbaseline (P < 0.05), when the mean surface density was 26 MFI units above baseline (T0) for the placebo condition, whereas it was only 5 MFI units above baseline for naltrexone; neither placebo nor naltrexone values differed significantly from the corresponding resting control value, which was 9 MFI units above its initial baseline.

The mean CD2 surface density of circulating CD56+ lymphocytes was reduced at each time point between T0,5 and T1 (Fig. 5A). Statistically, this drop of 8–14 MFI units did not reach significance until T4 in both placebo (P < 0.01) and naltrexone (P < 0.05) conditions. Differences between placebo and naltrexone conditions were not statistically significant.

**DISCUSSION**

The possible contribution of β-endorphin secretion to the exercise-mediated increase in peripheral blood NKCA remains controversial. Although a role for this endogenous opioid was demonstrated during short-term, exhaustive exercise (11), no effect was seen during short-term, moderate-intensity exercise under hypoxic conditions (30). Several components of the present study support the view that the endogenous release of β-endorphin does not mediate the enhancement of peripheral blood NKCA during prolonged moderate-intensity exercise.

First, NKCA increased by 85% relative to baseline levels during the first 60 min of exercise, despite the absence of any significant change in plasma β-endorphin levels over the same period. Furthermore, if β-endorphin were acting to enhance the cytolytic activity of NK cells, then measured levels of NKCA should have shown an increase during the last 60 min of exercise (when blood levels of β-endorphin were elevated) and measurements of per NKCA should have reflected this change. However, when NKCA was expressed on a per-NK cell basis, values during exercise were slightly lower than preexercise levels (P > 0.05).

Second, any direct stimulatory effect mediated by β-endorphin should be blocked by the potent, nonselective, opioid receptor antagonist naltrexone hydrochloride. However, this was not observed.

Third, the in vitro β-endorphin-mediated augmentation of NKCA has been attributed to accelerated kinetics of lysis (i.e., cell activation) and an enhanced effector-tumor cell conjugate formation (34). Both cellular activation and NK cell-target cell conjugate formation are dependent on initial binding events, in which cellular adhesion molecules such as LFA-1 and CD2 play a major role (44). Conceptually, a β-endorphin-mediated upregulation of LFA-1 or CD2 expression on NK cells could thus provide a mechanism by which exercise, secondary to the release of β-endorphin, increases NKCA. However, the present results speak against such an explanation of the in vivo data. Although the expression of LFA-1α and CD2 on peripheral blood NK cells changed during exercise, the mechanism of this change could not be attributed to a β-endorphin-mediated mechanism because the in vivo administration of naltrexone had no effect on this response. Nevertheless, the exercise-induced changes in LFA-1α and CD2 surface expression seem likely to influence the cytolytic response of NK cells during periods of physical exercise, and they thus deserve further examination.

Fourth, previous reports have suggested that the normal exercise-induced elevation in GH may be enhanced (10), inhibited (51), or unaffected (3) by opioid antagonism. Cortisol levels are typically enhanced, although more so in early recovery than during exercise (10). Such neuroendocrine changes could have secondary influences on NKCA. The in vivo effects of GH are unclear, but potentiating effects on human natural immunity have been described (7). Cortisol mediates both in vitro and in vivo inhibition of NKCA in humans (15, 16, 24). However, in the present study, naltrexone had no effect on circulating levels of either GH or cortisol, supporting the view that β-endorphin bears neither direct nor indirect responsibility for the exercise-induced increase in NKCA.

Given our essentially negative conclusions regarding the role of β-endorphin in modulating NKCA, it is
necessary to consider possible limitations of our experiments. The patterns of exercise differed from that of Fiatarone et al. (11) but nevertheless were sufficient to yield a substantial production of \( \beta \)-endorphin during the second hour of exercise. Nonexercise control values of plasma \( \beta \)-endorphin were not determined for reasons of cost. However, circadian variations in resting concentrations of plasma \( \beta \)-endorphin were not anticipated, because \( \beta \)-endorphin is not secreted tonically, but rather requires some stimulation of the nervous system to be formed and released (2). The bout of activity was also sufficient to induce the anticipated changes in the NKCA of peripheral blood (14), and cytolytic activity was measured by a well-accepted, standard technique (i.e., in vitro incubation of PBMC with K562 tumor cells at an effectortarget ratio of 50:1) (37). We may thus conclude that our results cannot be explained by an inadequate exercise stress or by problems in measuring NKCA.

Nor does it seem possible that the plasma concentrations of naltrexone were insufficient to block the effects of \( \beta \)-endorphin. A plasma naltrexone concentration of 2 ng/ml offers effective opioid blockade (54). Given effective blood levels of 2–9 ng/ml (Fig. 1A), the dose and timing of naltrexone administration must be judged as effective. However, the results could have been confounded by agonistic effects of naltrexone. Naloxone, an opioid antagonist with structural similarity to naltrexone, has either no direct effect (26, 29) or has donor-and dose-dependent stimulatory and inhibitory effects on the cytolytic function of NK cells (33). Until further study resolves this issue, any potential direct in vivo effects of naltrexone on NKCA remain speculative.

A further factor potentially confounding the present results is the possible involvement of naloxone-insensitive opioid receptors in the regulation of peripheral blood NKCA. Classically, opioid receptor binding involves the NH\(_2\)-terminal sequence Tyr-Gly-Gly-Phe of \( \beta \)-endorphin (1). However, lymphocytes also carry naloxone-insensitive, nonopioid receptors that bind the COOH-terminal residues of the molecule (23). Whetever opioid or nonopioid receptors are activated depends on the duration of exposure to and concentration of \( \beta \)-endorphin (42), which likely explains the inverted-U dose-response effect of \( \beta \)-endorphin. The removal of competitive binding between these receptors by a naltrexone-mediated blockade of classical opioid receptors might allow activation of nonopioid receptors. Binding of the COOH-terminal segments of \( \beta \)-endorphin to nonopioid receptors on lymphocytes would inhibit cytolytic function (55), providing a further mechanism for the slight trend to a postexercise depression of NKCA seen during naltrexone administration.

Alternative hypothesis. In a rested individual, the cytolytic activity of PBMCs is mediated almost exclusively by the NK cell subset; this accounts for 5–15% of the total PBMC population (45). The measured NKCA thus reflects both the concentration of NK cells and the cytolytic activity of each NK cell; thus increases in either factor could enhance NKCA. The most obvious alternative to the \( \beta \)-endorphin hypothesis, supported by our results, is that the exercise-induced increase in NKCA is secondary to an increased count of circulating NK cells (27). There is no simple relationship between changes in NK cell number and NKCA, but several researchers have suggested that the enhanced NKCA observed during a bout of physical exercise can be explained largely by an increased NK cell count rather than by an enhanced cytolytic capability per NK cell (4, 27, 49). In the present study, both the absolute and relative numbers of peripheral blood NK cells increased more than twofold during exercise, resulting in a 100% increase in the NK cell-to-K562 cell ratio (4:1 at rest compared with 9:1 during exercise). Furthermore, a tight correlation was found between the number of NK cells and the number of dead K562 in each assay tube (r = 0.82, P < 0.01), suggesting that increases in NK cell numbers could account for the demonstrated increase in NKCA.

Although not a primary objective of this study, postexercise changes in the study variables were examined. It is well accepted that exercise of sufficient intensity or duration can depress the peripheral blood NKCA postexercise (14). The trend for naltrexone to affect the postexercise NKCA may thus be an important observation. When naltrexone and placebo data were compared with the 2-h postexercise control value, there was a 32% drop in percent lysis in the naltrexone condition (P < 0.01) but only a 20% drop in percent lysis during the placebo condition (P > 0.05). This may reflect a release of cortisol in parallel with \( \beta \)-endorphin. ACTH is released from the pituitary gland concomitantly with \( \beta \)-endorphin (21), and it in turn stimulates the release of cortisol. In agreement with this, \( \beta \)-endorphin and cortisol levels showed a similar kinetic response in the present study. Cortisol is potentially inhibitory to the cytolytic activity of NK cells both in vitro and in vivo (15, 16, 24); however, addition of physiological concentrations of \( \beta \)-endorphin can ameliorate the in vitro cortisol-induced inhibition of human NKCA (17). Conceptually, \( \beta \)-endorphin release during the later stages of prolonged moderate physical exercise may thus prevent the overshoot of glucocorticoid-dependent immunosuppression, specifically counteracting the negative effects of elevated cortisol levels on NKCA. Therefore, the removal of the \( \beta \)-endorphin-mediated, counter-regulatory mechanism may have tipped the balance between these two opposing modulatory hormones in favor of cortisol-induced immunosuppression. It is already known that peripheral blood lymphocytes down-regulate their sensitivity and binding capacity for glucocorticoids after strenuous exercise (8, 50). The exercise-induced release of \( \beta \)-endorphin may provide a further mechanism to prevent any untoward effects of elevated blood levels of cortisol. Although naltrexone did not affect the release of cortisol at the time points examined in this study, the previously described action of opioid antagonists in augmenting cortisol release during early recovery (i.e., 0–60 min) from moderate- to high-intensity exercise (10, 20) may have further promoted cortisol-induced immunosuppression.
It must be stressed that our study has not ruled out the possibility that the potentiating effect of β-endorphin may require its concomitant presence during the cytolytic process. It has been well established that the in vitro exposure of NK cells to β-endorphin can indeed promote NKCA in a dose-dependent fashion, perhaps stimulating what actually happens in vivo. In this study, the culture of NK cells ex vivo, in the absence of β-endorphin, may diminish or negate its putative potentiating effect. This postulate appears to reconcile the apparent difference between the cytolytic stimulatory effect of β-endorphin in direct contact with the effector cells during the culture period and the lack of apparent potentiation observed in this study, in which the effector cells were previously exposed to an exercise-induced physiological concentration of β-endorphin but the neu- ropeptide was absent during the NK cell-mediated cytolytic process.

With the assumption that confounding mechanisms can be excluded, the results of this study do not support the hypothesis that β-endorphin is an important mediator of the acute augmentation of peripheral blood NKCA during prolonged, moderate-intensity exercise because 1) NKCA is enhanced during the early stage of prolonged moderate-intensity exercise despite there being no concomitant increase in plasma β-endorphin, 2) a later increase in plasma β-endorphin does not further increase NKCA, and 3) the nonselective opioid antagonist naltrexone had no statistically measurable effect on the response of NKCA or on the regulation of other neuroendocrine or cell adhesion factors that could influence NKCA. We conclude that the primary explanation for the exercise-induced changes in NKCA during prolonged exercise is a concomitant change in NK cell count with essentially no change in per NKCA. On the other hand, the present results do not exclude a possible regulatory effect of β-endorphin on NKCA early in the postexercise period.

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