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


β-Endorphin and natural killer cell cytolytic activity during prolonged exercise. Is there a connection? Am. J. Physiol. 275 (Regulatory Integrative Comp. Physiol. 44): R1725–R1734, 1998. 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 is 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 (preexercise) levels 2 h postexercise. CD3+CD16+CD56+ NK cell counts and NKCA were significantly (P < 0.05) elevated at each 30-min interval of exercise compared with correspondingly timed resting control values. However, there were no differences 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

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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 (intercellular adhesion molecule 1 and LFA-3) play a major role during the recognition, conjugation, and cytolysis 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.

Experimental design. This study was composed of five laboratory visits: 1) clinical, physical, and anthropometric assessment, 2) a nonexercise, resting control trial, and 3) three double-blind exercise tests ordered according to a random block design (placebo, naltrexone, and indomethacin). For the present purpose, only the resting control, placebo, and naltrexone trials will be considered. The trial involving administration of indomethacin has been described by Rhind et al. (42a).

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 pedaling 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 (Insyte, 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 pedaling 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 (Polar Vantage XL) were monitored at 15-min intervals 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 hemocencentration. 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, T0.5, T1, T1.5, T2, 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 Hematology 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× isotonic PBS containing 0.1% sodium azide. Stained-
CD2 on CD56
(BDIS) for mean fluorescence intensity (MFI) of CD11a and list mode data were analyzed with CELLQuest software. Stained-cell suspensions were analyzed for fluorescence on a mononuclear cell (PBMC) suspensions were stained with higher target cell concentration (43). With this method, the expedite data acquisition, we modified this method by using a cells was assessed by an in vitro flow cytometric assay (22). To
analyzed using CellQUEST software (BDIS). 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 FACSscan flow cytometer (BDIS). Acquired list mode data were analyzed with CellQuest software (BDIS) for mean fluorescence intensity (MFI) of CD11a and CD2 on CD56+ 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 centrifugation for 5 min (400 g, 20°C); they were then resuspended to a final concentration of 107 cells/ml. K562 tumor cells (targets, at 2 x 105 cells/ml) were gently mixed with 100 µl of PKH26-labeled K562 tumor cells (targets, at 2 x 105 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% CO2). 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 FACSscan flow cytometer and CellQuest software (BDIS). PKH26+ target cells were defined and live gated via a histogram of F12 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 125I RIA (Incstar, Stillwater, MN). Total plasma cortisol concentrations were determined in duplicate using a competitive solid-phase 125I 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-β-naltrexol analyses. Plasma samples (150 µl) 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 pentfluoropropionic anhydride for 45 min at 80°C. Excess pentfluoropropionic anhydride was removed by drying with N2, and 1-ml aliquots were injected into a Finnigan TSQ-700 GC-MS/MS capillary column equipped with 15m DB-5 (J and W Scientific, low load, 0.25 mm ID). The GC injector was held at 250°C; the column was ramped from 140°C to 250°C at 25°C/min and then held at this temperature for 10 min. The mass spectrometer conditions were an ion source at 110°C, and reagent gas was optimized using negative chemical ionization fragment m/z 633 of pentfluorotributyl amine, 70 eV, ion current 200 mA, single ion monitoring m/z 779 and m/z 782 for 6-β-naltrexol pentfluoropropionic anhydride (PF) and m/z 761 and m/z 764 for naltrexone and D3-naltrexone, with dwell times of 30 ms. The calibration curves were constructed by spiking blank plasma with 0.5, 2.5, 10, and 20 ng/ml of naltrexone and 5 ng/ml of D3 naltrexone internal standard (I.S.) and 5, 50, 100, 200 ng/ml of 6-β-naltrexol and 50 ng/ml of D3 β-naltrexol I.S. Linear

β-ENDORPHIN, NK CELL CYTOLYTIC ACTIVITY, AND EXERCISE

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 x 107 cells/ml) were gently mixed with 100 µl of PKH26-labeled K562 tumor cells (targets, at 2 x 105 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% CO2). The assay was stopped by addition of cold cell wash to the cultures. Samples were placed on ice until same-day analysis.

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regressions and sample concentration calculations were done using the spreadsheet Microsoft Excel. Statistical analyses. Analyses were performed using the Statistical Package for the Social Sciences (SPSS/PC+, Windows version 7.0). Data are presented as means ± SE unless otherwise noted. To determine circadian effects, we analyzed resting control data using a one-way (time) repeated-measures (RM) ANOVA across five time points (T₀, T₁, T₂, T₃, and T₄). A two-way (condition × time) RM ANOVA was used to determine the main effects of condition (control vs. placebo vs. naltrexone), time (T₀, T₀.5, T₁, T₁.5, T₂, T₃, and T₄), 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, P = 0.76), time (F₃,₂₇ = 0.17, P = 0.91), and condition × time interaction (F₃,₂₇ = 0.67, P = 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 6-β-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 6-β-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 conditions demonstrated a significant main effect of time (F₂,₂₃ = 3.29, P < 0.05); counts at T₂₄ (0.23 ± 0.03 × 10⁸ cells/l) were significantly greater (P < 0.05) than counts at T₀ (0.17 ± 0.02 × 10⁸ cells/l).

Over the three conditions, monocyte counts (data not shown) showed significant main effects of condition (F₁,₀.₆₄ = 4.32, P < 0.05) and time (F₂,₄₂₁ = 21.84, P < 0.001), with a condition × time interaction (F₄,₀.₆₂ = 5.73, P < 0.001). Relative to the corresponding control data, the moderately intensive placebo cycling protocol elevated the total circulating monocyte count by 104% (P < 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 (F₁,₁₃₃₆ = 42.92, P < 0.001) and time (F₁,₈₅₈ = 40.44, P < 0.001) and a condition × time interaction (F₃,₂₂₈₉ = 28.85, P < 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 steady-state 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 (F₂,₆₂₃₂ = 81.86, P < 0.001) and a significant interaction effect (F₃,₂₇₂ = 16.33, P < 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, P < 0.01) but fell 28% below resting control values at T₄ 2 h postexercise (P < 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
significant reduction ($P < 0.05$) relative to corresponding control values, whereas the naltrexone data did not.

Measures of NKCA at baseline ($T_0$) 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 ($T_0$) values (Fig. 2C). This made the trend to differences between placebo and naltrexone conditions more apparent. Relative to corresponding baseline ($T_0$) values, there was a significant ($P < 0.01$) 33% drop in percent lysis at 2 h postexercise ($T_4$) 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 $1.0 \times 10^6$ 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 $1.0 \times 10^6$ PBMCs showed only minor shifts (interaction effect; $F_{3,73,37} = 1.54$, $P = 0.21$); 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 ($T_0$), over 9:1 during exercise ($P < 0.01$), and between 4 and 5:1 postexercise ($T_4$ and $T_{24}$) 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 a baseline ($T_0$) value of $7.3 \times 10^{-2}$ dead K562 cells to below $6.0 \times 10^{-2}$ dead K562 cells during exercise, while increasing to $6.3 \times 10^{-2}$ dead K562 cells at $T_4$ and $T_{24}$. 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 $T_0$ and $T_2$) 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; F1,6,148 = 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 (F1,8,12,8 = 4.34, P < 0.05) and time (F3,2,22,0 = 3.15, P < 0.05), with a significant interaction effect (F6,8,47,6 = 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 (F2,2,12,1 = 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 (T2). 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 the decrease in CD11a density at T1.5 (P < 0.05) in the naltrexone condition compared with placebo.

Significant increases were observed in CD2 expression at T1.5 and T2 for both placebo and naltrexone conditions (P < 0.05). However, the increase was larger and more persistent in naltrexone (Fig. 5B). Total plasma cortisol concentrations (Fig. 4C) showed significant main effects of condition (F1,8,12,8 = 4.34, P < 0.05) and time (F3,2,22,0 = 3.15, P < 0.05), with a significant interaction effect (F6,8,47,6 = 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 (F2,2,12,1 = 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.

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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 T₄ (Fig. 5B). 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 vivo incubation of PBMC with K562 tumor cells at an effector:target 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 naltrexone-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 naltrexone-insensitive, nonopioid receptors that bind the COOH-terminal residues of the molecule (23). Whether 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 potently 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 cytotytic 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 cytotytic stimulatory effect of β-endorphin in direct contact with the effecter cells during the culture period and the lack of apparent potentiation observed in this study, in which the effecter cells were previously exposed to an exercise-induced physiological concentration of β-endorphin but the neuropeptide was absent during the NK cell-mediated cytotytic 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 the absence of NKCA, and 2) a later increase in plasma β-endorphin does not further increase NKCA, and 3) the nonselective opioid antagonist naloxone 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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Address for reprint requests: G.A. Gannon, Human Protection and Performance Section, Defence and Civil Institute of Environmental Medicine, 1133 Sheppard Ave. West, Toronto, Ontario, Canada M3M 3B9.

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