Carbohydrate intake during endurance exercise increases natural killer cell responsiveness to IL-2

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Carbohydrate intake during endurance exercise increases natural killer cell responsiveness to IL-2. J Appl Physiol 96: 271–275, 2004. First published September 12, 2003; 10.1152/japplphysiol.00585.2003.—The purpose of this study was to evaluate the effect of high-intensity endurance exercise and carbohydrate consumption on in vitro responsiveness of natural killer (NK) to IL-2 (2.5 U/ml for 24 h). Thirteen male subjects (18–26 yr old; peak O2 consumption = 59.79 ± 5.13 ml·kg⁻¹·min⁻¹) were recruited to complete two 1-h (75–80% peak O2 consumption) cycling trials in a random counterbalanced order: carbohydrate (CHO) and placebo (Pla). Venous blood samples were collected before (Pre), immediately (Post), 2 h (2H), and 4 h (4H) after exercise. All resting samples were taken after 15 min of seated rest. NK (CD3⁺/CD56⁻), activated NK (CD3⁺/CD56⁺/CD69⁺), helper T cell (Th; CD3⁺/CD4⁺), and cytotoxic T cell (Tc; CD3⁺/CD8⁺) number were measured by using flow cytometry. NK cell activity (NKCA) was determined by using both a 51Cr release assay (NKCA-51) and activated NK cell number (NKCA-69). Immune system variables were not different between CHO and Pla, with the exception of NK cell responsiveness to IL-2, where Post (116.2%) and 4H (48.4%) was significantly greater in CHO (P < 0.05). NK, Th, and Tc were significantly higher Post (40.7, 102.7, and 82.0%, respectively) and lower at 2H (−51.9, −53.3, and −53.2%, respectively) than Pre (time effect). 4H was not different from Pre for NK, Th, and Tc. NKCA was significantly lower 2H (NKCA-51, NKCA-69) and 4H (NKCA-69) than Pre. CHO consumption during exercise did not prevent disruptions in unstimulated immune system function, but it did enhance NK responsiveness to IL-2.

natural killer cell activity; flow cytometry; CD69; cyclists; whole blood natural killer assay; interleukin-2

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edge, no published research has identified a mechanism to explain the effect of carbohydrate consumption during exercise on NK cell responsiveness to IL-2. The primary purpose of this study was to determine the effects of carbohydrate consumption during exercise on NK cell responsiveness to IL-2 in vitro. A secondary purpose was to compare NKCA, measured by using a whole blood $^{51}$Cr release assay, to CD69$, measured by flow cytometry.

**METHODS**

**Subjects.** Thirteen male subjects (18–24 yr) volunteered (Table 1) after the risks and benefits of participation were explained and they completed university-approved consent and medical history forms (approval no. 01-564). Subjects reported to the laboratory at least 7 days before their first experimental trial and completed an incremental, discontinuous $\text{VO}_2$ peak test on an electrically braked cycle ergometer (Lode). The test consisted of 2-min exercise stages, separated by 30 s of rest. Stages were completed until subjects indicated that they wished to stop. Respiratory gases and heart rate were measured throughout the test by automated analysis (Truemax 2800; Parvomedics, Salt Lake City, UT) and telemetry (Polar, Port Washington, NY), respectively.

**Trial conditions.** Subjects arrived at the laboratory between 0500 and 0700 and completed one of two exercise trials in a random, counterbalanced order to account for order effects: carbohydrate trial (CHO; Gatorade, Quaker Oats, Barrington, IL) and placebo trial (Pla; water). The subjects were instructed to consume their normal mixed diet on the day before each experimental trial. Exercise consisted of 30 min of continuous cycling on a fixed-belt bicycle ergometer (Lode). The test consisted of 2-min exercise stages, separated by 30 s of rest. Stages were completed until subjects indicated that they wished to stop. Respiratory gases and heart rate were measured throughout the test by automated analysis (TrueMax 2800; Parvomedics, Salt Lake City, UT) and telemetry (Polar, Port Washington, NY), respectively.

**Blood collection.** Venous blood samples (20 ml) were collected from a peripheral arm vein into evacuated tubes treated with either sodium heparin (Becton-Dickinson) or EDTA (Becton-Dickinson) before exercise (Pre), and immediately (Post), 2 h (2H), and 4 h (4H) after exercise. All blood samples, with the exception of Post, were taken after a 15-min seated rest. EDTA tubes were prechilled at $4^\circ\text{C}$ before blood collection and then were centrifuged within 30 min of collection to isolate plasma. Sodium heparin-treated blood was stored at room temperature on a rocker until the measurement of NKCA and leukocyte number.

**Leukocyte number.** To determine total leukocyte count, whole blood (20 $\mu$l) was added to 10 $\text{ml}$ of sterile phosphate-buffered saline (pH = 7.2) in a polystyrene cuvet (Fisher Scientific). Five drops of manual lysing solution (Fisher Scientific) were added, and the resultant solution was allowed to incubate at room temperature for 30 s. Triplicate total leukocyte cell counts were determined on a particle counter (Beckman-Coulter Z$^2$, Miami, FL).

**Flow cytometry.** Sodium heparin-treated whole blood (100 $\mu$l) was pipetted into the bottom of three 12 $\times$ 75 mm polystyrene tubes (Sarstedt). The following antibodies (20 $\mu$l each) were added to the first tube: CD3-fluorescein isothiocyanate (FITC; Beckman-Coulter, clone UCHT1), CD56-phycocerythrin (PE; Beckman-Coulter, clone N901), and CD69-phycocerythrin-cyanine 5 (PECy5; Beckman-Coulter, clone TP1.55.3); and to the second tube: CD4-FITC (Beckman-Coulter, clone 13B8.2) and CD8-PE (Beckman-Coulter, clone B9.11). A three-color (FITC, PE, and PECy5) isotype control antibody (Beckman-Coulter) was added to the third tube and used to adjust compensation for the combination of FITC, PE, and PECy5 fluorochromes present in the analysis. The whole blood antibody mixtures were incubated at room temperature for 30 min in the dark. After incubation, samples were processed for analysis by using an automated system (Immunoprep, Beckman-Coulter). Initial gates were established for lymphocytes on the basis of forward- and side-scatter light. Secondary gates were established for NK cells for CD3$^+$ and CD56$^+$, activated NK cells (NKCA-69, CD3$^+$ /CD56$^+$ /CD69$^+$), Th (CD3$^+$ /CD8$^-$), and Tc (CD3$^+$ /CD8$^+$). Flow cytometric analysis was completed by using a Beckman-Coulter XL-MCL cytometer equipped with an air-cooled argon laser (excitation wavelength = 488 nm).

**Stimulation of whole blood.** All cell culture work was performed in a class II laminar flow hood (Labconco, Kansas City, MO) by using aseptic techniques. Within 2 h of blood collection, 100-$\mu$l aliquots of heparin-treated whole blood were carefully added to each of 24 wells (2 rows) of flat-bottom sterile 96-well microplates (Corning). Either 50 $\mu$l of culture medium (first 12 wells) or culture medium supplemented with IL-2 (second 12 wells) (final concentration: 2.5 $\mu$/ml; Sigma-Aldrich, St. Louis, MO) were added. Optimal IL-2 concentration was determined on the basis of a lot-specific titration curve performed before the study (data not shown). The plate was covered and incubated for 24 h ($37^\circ\text{C}, 5\% \text{CO}_2$, humidified environment). After incubation, plates were removed from the incubator and kept at room temperature until analysis of NKCA with the use of a standard $^{51}$Cr release assay. NK cell responsiveness was determined by subtracting unstimulated NKCA from IL-2-stimulated NKCA.

**Whole blood NKCA.** Analysis of NKCA (unstimulated NKCA-51 and IL-2 stimulated) was completed by using the method described previously by Baron et al. (2). Human chronic myelogenous leukemia cells (K562; CCL-243; ATCC, Manassas, VA) were labeled with $^{51}$Cr and suspended in culture medium only or IL-2-stimulated whole blood samples. Spontaneous (100 $\mu$l of culture medium) and total lysis (100 $\mu$l of 1% Triton X) was determined in triplicate for each of the four target (K562) cell concentrations.

**Addition of target cells.** The microplates were centrifuged for 10 min (100 g) and incubated for 4 h ($37^\circ\text{C}, 5\% \text{CO}_2$, humidified environment). Chilled Hanks’ balanced salt solution (75 $\mu$l) was added to stop the reaction. The plates were centrifuged for 10 min (100 g), and 100 $\mu$l of the resultant cell-free supernatant were counted on a gamma counter (5 min per tube, Packard Instruments). Percent lysis for each target cell concentration was determined by using the following equation

\[
\%\text{Lysis} = \frac{\text{(sample cpm} - \text{control cpm})}{\text{(total cpm} - \text{control cpm})}\times 100
\]

where cpm is counts per minute. NKCA was expressed on a 1 effector to 1 target cell basis by using kinetic plotting (2, 8, 17).

**Statistical analysis.** All blood measurements were analyzed by using a 2 (CHO and Pla) $\times$ 4 (Pre, Post, 2H, and 4H) factor ANOVA.

<table>
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<th>Characteristic</th>
<th>Mean</th>
<th>SD</th>
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<td>Height, cm</td>
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<td>6.13</td>
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<td>5.01</td>
<td>64.80–75.00</td>
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<tr>
<td>Percent body fat, %</td>
<td>7.87</td>
<td>3.20</td>
<td>4.22–12.77</td>
</tr>
<tr>
<td>$\text{VO}_2$ peak, ml $\text{kg}^{-1}\text{min}^{-1}$</td>
<td>59.79</td>
<td>5.13</td>
<td>50.30–69.30</td>
</tr>
</tbody>
</table>

$\text{VO}_2$ peak, peak $\text{O}_2$ consumption.
with repeated measures on both factors. Cardiovascular measurements were compared by using a 2 (CHO and Pla) × 3 (5, 30, and 60 min of exercise) factor ANOVA with repeated measures on both factors. Before analysis, assumptions of normality and constant variance were confirmed by using quantile-quantile and residual plots, respectively. Significant $P$ values ($<0.05$) were adjusted by using the Huynh-Feldt method to account for repeated measures. When significance was found, a Student’s $t$-test with Bonferroni correction for multiple comparisons was used to determine the location of significance. All values are presented as means ± SE. A Pearson’s bivariate correlation was used to evaluate the significance of the relationship between NKCA-51 and NKCA-69. Statistical analysis was completed by using SPSS 10.1 (SPSS, Chicago, IL).

RESULTS

Cardiovascular measurements. $\dot{V}O_2$ and heart rate were not significantly different between CHO (3.33 ± 0.05 l/min and 170.1 ± 1.8 beats/min, respectively) and Pla (3.34 ± 0.05 l/min and 172.4 ± 1.8 beats/min, respectively). $\dot{V}O_2$ (3.33 ± 0.04 l/min) did not change significantly over the course of the exercise bout, whereas heart rate ($F = 14.559, P < 0.001$) increased (5 min = 166.2 ± 4.3 beats/min; 60 min = 173.8 ± 3.6 beats/min). Plasma glucose was significantly greater Post (6.29 ± 0.28 mM) and 4H (5.87 ± 0.33 mM) during CHO compared with similar time points in Pla (Post = 5.34 ± 0.34 mM; 4H = 4.37 ± 0.15 mM) ($F = 10.479, P < 0.001$).

Leukocyte responses. Total leukocyte count was significantly elevated Post (88.1%), 2H (63.9%), and 4H (47.9%) above preexercise values (main effect for time) ($F = 14.697, P < 0.001$). Recovery samples (Post, 2H, and 4H) were not significantly different from one another. The total number of Th ($F = 29.353, P < 0.001$; Fig. 1A), Tc ($F = 37.514, P < 0.001$; Fig. 1B), and NK cells ($F = 44.717, P < 0.001$; Fig. 1C) was significantly higher at Post (40.7, 102.7, and 82.0%) and significantly lower at 2H (−51.9, −53.3, and −53.2%) than Pre. After 4H recovery, the total numbers of Th, Tc, and NK cells were not different than Pre.

NKCA. Evaluation of NKCA by activated NK cell number (NKCA-69) and a $^{51}$Cr release assay (NKCA-51) did not reveal a significant main effect for drink or an interaction between drink and time. A significant main effect for time was found for NKCA-69 where Post was increased (188.7%), 2H was decreased (−51.9%), and 4H was decreased (−38.4%) compared with Pre ($F = 61.689, P < 0.001$; Fig. 2A). 2H and 4H samples were not significantly different from each other but were significantly lower than Post. NKCA-51 was significantly greater at Post (289.9%) and lower at 2H (−40.0%) than Pre sample ($F = 41.691, P < 0.001$; Fig. 2B). Pre and 4H samples in both tests were similar. A Pearson’s bivariate correlation ($n = 103$) revealed a significant positive relationship between NKCA-69 and NKCA-51 ($r = 0.67, P < 0.001$).

NK cell responsiveness to IL-2 in vitro. A significant interaction was found between drink and time ($F = 6.215, P = 0.010$; Fig. 3) for IL-2-stimulated NK cell responsiveness. IL-2 responsiveness was higher Post (116.2%) and 4H (48.4%) during CHO than similar time points in Pla.

DISCUSSION

The primary finding of this study was that carbohydrate consumption during 1 h of endurance cycle ergometer exercise enhanced NK cell responsiveness to IL-2 in vitro but did not influence unstimulated NKCA. NKCA increased immediately Post and then fell below preexercise values at 2H (NKCA-51 and NKCA-69) and 4H (NKCA-69). Consumption of carbohydrate during exercise did not change circulating NK cell, Th, or Tc number compared with Pla. NKCA-69 has been reported as a sensitive measure of NKCA because it examines the cell surface. The exercise bout, regardless of supplement, produced similar responses for NKCA-51 and NKCA-69, suggesting that exercise, but not carbohydrate, affects unstimulated NKCA.

The literature has established that carbohydrate consumption during endurance exercise (10, 17, 20, 23), but not carbohy-
endurance (75% of maximal V\textsubscript{\dot{O}_2 peak}) in carbohydrate and placebo trials. Exercise consisted of 1 h of cycle ergometer exercise at 75–80% of \textsubscript{\dot{O}_2 peak}. Exercise consisted of 1 h of cycle ergometer exercise at 75–80% of \textsubscript{\dot{O}_2 peak}.

Fig. 2. A: means ± SE for activated NK cell number (NKCA–69, CD3\(^+\)/CD56\(^+\)/CD16\(^+\)) in carbohydrate and placebo trials. B: means ± SE for NK cell activity measured by using a whole blood \textsuperscript{51}Cr release assay (NKCA–51) in carbohydrate and placebo trials. Exercise consisted of 1 h of cycle ergometer exercise at 75–80% of \textsubscript{\dot{O}_2 peak}. 1: E:1T, 1 effector:1 target. \*, significantly different from Pre (P < 0.05).

Contrary to Nieman et al., we did not find a difference in either NK cell number or activity when carbohydrate was consumed. Our findings are supported by Mitchell et al. (17), who reported that carbohydrate consumption during exercise did not influence NK cell number or unstimulated NKCA. In the present study, carbohydrate consumption increased in vivo NK cell responsiveness to IL-2, which suggests that a combination of exercise and carbohydrate consumption may enhance the number of NK cells that are responsive to T1 cytokines in blood. Previous studies from our laboratory (16) and others (26) have reported that exercise stress produces changes in NK cells above that observed after a resting control trial.

Activation of T1 pathways results in release of the cytokines IL-2 and IFN-\(\gamma\), which activate a variety of leukocytes, including macrophages, neutrophils, and NK cells (7). Similar to T cells, NK cells can be divided into both type 1 (NK1) and type 2 cells (15). T1 cells may favor recruitment of NK1 cells because this subtype of NK cell has been reported to be more responsive to T1 cytokines (15, 25). Ibfelt et al. (13) reported that 1.5 h of downhill running (75% of maximal \textsubscript{\dot{V}_O2}) caused a significant decline in the percentage of CD8\(^+\) cells producing IFN-\(\gamma\) (Tc producing T1 cytokines) and no change in CD8\(^+\) cells producing IL-4 (Tc2). This finding was supported by that of Steensberg et al. (30), who reported that 2.5 h of treadmill running (75% of maximal \textsubscript{\dot{V}_O2}) caused a significant decrease in the percentage of circulating Th cells producing T1 cytokines immediately and 2 h after exercise. The difference in IL-2 responsiveness, observed in the present study, could be explained based on T1/T2 T cell balance, but we are unable to determine whether this is the only contributing factor. Ibfelt et al. (13) and Steensberg et al. (30) employed exercise protocols that were more severe than the present study (1 h at 75–80% of \textsubscript{\dot{V}_O2 peak}), but other studies (16, 17, 26) have reported that exercise similar to ours caused a significant degree of physiological stress. Other physiological factors (i.e., hypoglycemia) have also been reported to enhance exercise-induced release of stress hormones, which may affect T cell responses (13, 29, 30).

Although hypoglycemia has been reported to enhance exercise responses, others have reported that a low-carbohydrate diet independent of exercise does not significantly disrupt the immune system (3, 18). Unfortunately, these studies did not specifically examine NKCA; therefore, it is possible that carbohydrate consumption, independent of exercise, may affect NKCA. Short-term exposure (24 h) to IL-2 in vitro is known to increase mRNA expression of perforin and IFN-\(\gamma\), increase cell-surface expression of adhesion molecules, and elevate NKCA (5). Emotional distress has been demonstrated to cause similar elevations in plasma stress hormones observed with exercise. Cohen et al. (6) reported that emotional distress caused a significant reduction in T1 cytokines and NKCA. Although stress hormones could have influenced our findings, it is unlikely that they are the only contributing factor.

Although no direct measures of T1/T2 balance or NK cell subtype were made in the present study, exercise-induced enhancement of T2 pathways via stress hormones and a reduction in NK1 cells may partially explain the reduction in NKCA during recovery that we observed and may provide additional support for the “open window” hypothesis. Others have reported that epinephrine regulates NK cell adhesion markers, which in turn control the response to T1 cytokines (5). Therefore, although the present study and others (17, 20) have shown that carbohydrate administration during exercise does not improve unstimulated NKCA, NK cell response to IL-2 was significantly improved.

A variety of techniques have been used to measure NKCA. Flow cytometric analysis is popular because early activation markers can be quickly assessed and it may be more sensitive than traditional in vitro assays (26). Whole blood analysis is a popular alternative to methods employing isolated mononuclear cells because it maintains the blood borne milieu, which...
likely regulate NKCA during recovery from exercise (4, 9, 17). Also, density gradient isolations can result in a significant loss of leukocyte fractions and may impact functional capacity (11). Although additional research is needed to evaluate factors in the blood milieu, NKCA-69 (cell surface) and NKCA-51 (whole cell) responded similarly to exercise stress. The methods utilized in the present study were consistent with the literature; however, in vitro measurements do not necessarily reflect the in vivo immune system response. Furthermore, based on the present data, we are unable to determine whether acute shifts in leukocyte populations or function pose any health consequences.

In conclusion, carbohydrate consumption combined with exercise enhanced NK cell responsiveness to IL-2 in vitro but did not influence unstimulated NKCA, NK, Th, or Tc number. Further research is needed to determine additional factors that may regulate T1/T2 balance and its effect on NKCA. These studies may help determine the nature of the relationship between NK cells and other leukocyte subsets after high-intensity endurance exercise when carbohydrate is consumed.

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GRANTS

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REFERENCES


