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

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McFarlin, Brian K., Michael G. Flynn, Laura K. Stewart, and Kyle L. Timmerman. Carbohydrate intake during endurance exercise increases natural killer cell responsiveness to IL-2. J Appl Physiol 96: 271–275, 2004.—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·min) 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+), 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 (−5.1, −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

HIGH-INTENSITY ENDURANCE EXERCISE causes significant disruptions in normal leukocyte trafficking and function (19, 27). Natural killer (NK) cell activity (NKCA) is one measure of the activity of the innate immune system. Although the precise mechanism is still widely debated, several authors have reported that NKCA declines in the hours after strenuous exercise (19, 24). The magnitude of the immune response is altered by the degree of physiological stress applied during exercise, which is dependent on several factors, including intensity, duration, and internal stimuli (i.e., blood glucose levels) (10, 17, 20). High-intensity [70–80% peak O2 consumption (VO2)] (VO2 peak), long-duration (1–2 h) endurance exercise provides the greatest stimulus of the immune system via release of “stress hormones” (i.e., glucocorticoids and catecholamines) (19). Some authors suggest that carbohydrate consumption during strenuous exercise may blunt the release of stress hormones and their potential impact on the immune system (21, 23).

In addition to circulating stress hormones, cytokines released from activated T cells may play a significant role in recruitment of NK cells (15) and regulation of NKCA (12, 31). Type 1 T cell (Th1) cytokines, interferon (IFN)-γ, and interleukin (IL)-2 increase NKCA (31), whereas type 2 T cell (Th2) cytokines IL-4 and IL-10 block the action of Th1 cytokines (12). Das et al. (7) reported that coculture of NK cells in the presence of T cells (CD3+) enhanced NKCA over that observed after coculture with other leukocyte subsets (B cells or macrophages). Arman et al. (1) reported that IL-2 released from T cells enhanced NKCA and prevented glucocorticoid-induced apoptosis of NK cells. Endurance exercise stimulates an increase in circulating NK cells responsive to IL-2, suggesting that Th1 cytokines play an important role in NK cell function (24). This finding was supported by Shephard et al. (28), who reported that physically demanding exercise and the resultant drop in NKCA during recovery are related to a reduction in cell-surface expression of IL-2 receptors on NK cells. High-intensity endurance exercise has been reported to cause a significant reduction in T cell number during recovery from exercise (13, 30). Cytotoxic T cells (Tc) producing T1 cytokines and, to a lesser degree, helper T cells (Th) producing T1 cytokines decline after exercise, resulting in T2 dominance (30). Kohut et al. (14) reported that fatigue during exercise (~2.5 h of wheel running) in mice caused a significant reduction in T1 cytokines and NKCA for up to 2 days after exercise. Although the exact mechanisms underlying T2 dominance after exercise are not fully understood, they may be related to the release of cytokines IL-4 and IL-10 block the action of T1 cytokines (12), whereas type 2 T cell (Th2) cytokines IL-4 and IL-10 block the action of Th1 cytokines (12). Das et al. (7) reported that coculture of NK cells in the presence of T cells (CD3+) enhanced NKCA over that observed after coculture with other leukocyte subsets (B cells or macrophages). Arman et al. (1) reported that IL-2 released from T cells enhanced NKCA and prevented glucocorticoid-induced apoptosis of NK cells.

Nieman et al. (20) reported that carbohydrate consumption during endurance exercise (1-h at 75% of maximal VO2) increased NK cell number but not unstimulated NKCA during recovery. Other studies (3, 18) have reported that a high-carbohydrate diet does not significantly affect the immune system. To our knowledge, no present research has evaluated the effect of carbohydrate consumption and exercise on in vitro NK cell responsiveness to a stimulus (i.e., IL-2). We hypothesize that carbohydrate consumption during exercise and recovery will not change unstimulated NKCA but will improve IL-2 responsiveness. To our knowl-
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 ⁵¹Cr 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 VO₂ peak test on an electronically 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 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°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.

**Lyseocyte number.** To determine total leukocyte count, whole blood (20 µl) was added to 10 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 Z₂, Miami, FL).

**Flow cytometry.** Sodium heparin-treated whole blood (100 µl) was pipetted into the bottom of three 12 × 75 mm polystyrene tubes (Sarstedt). The following antibodies (20 µl 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 TP.1,55.3); 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 (CD3⁺/CD56⁺), activated NK cells (NKCA-69, CD3⁺/CD56⁺/CD69⁺), Th (CD3⁺/CD56⁻), and Te (CD3⁺/CD56⁻). All 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 completed in a class II laminar flow hood (Labconco, Kansas City, MO) by using aseptic techniques. Within 2 h of blood collection, 100-µl 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 µl of culture medium (first 12 wells) or culture medium supplemented with IL-2 (second 12 wells) (final concentration: 2.5 U/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°C, 5% CO₂, 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 ⁵¹Cr release assay. NK cell responsiveness was determined by subtracting unstimulated NKCA from IL-2-stimulated NKCA.

**Whole blood NKCA.** Analysis of NKCA (unstimulated NKCA-51Cr 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 ⁵¹Cr and suspended at the following concentrations: 2.00, 1.00, 0.50, and 0.25 × 10⁶ cells/l. The microplates were centrifuged at 200 g × 3 min before being loaded into the 96-well microplates. One hundred microliters of culture medium was added to triplicate (second 12 wells) (final concentration: 2.5 U/ml; Sigma-Aldrich, St. Louis, MO). K562 cells were added to sets of 3 wells (second 12 wells) in triplicate (second 12 wells) (final concentration: 2.5 U/ml; Sigma-Aldrich, St. Louis, MO) supplemented with IL-2 (second 12 wells) (final concentration: 2.5 U/ml; Sigma-Aldrich, St. Louis, MO). The following antibodies (20 µl each) were added to the first tube: CD3-phycoerythrin (PE; Beckman-Coulter, clone UCHT1), CD56-phycoerythrin-cyanine 5 (PECy5; Beckman-Coulter, clone TP.1,55.3); 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 (CD3⁺/CD56⁺), activated NK cells (NKCA-69, CD3⁺/CD56⁺/CD69⁺), Th (CD3⁺/CD56⁻), and Te (CD3⁺/CD56⁻). All flow cytometric analysis was completed by using a Beckman-Coulter XL-MCL cytometer equipped with an air-cooled argon laser (excitation wavelength = 488 nm).

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

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 2 (CHO and Pla) × 4 (Pre, Post, 2H, and 4H) factor ANOVÀ

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Table 1. Subject characteristics for 13 male subjects

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Mean</th>
<th>SD</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>20.38</td>
<td>1.61</td>
<td>18.00–24.00</td>
</tr>
<tr>
<td>Height, cm</td>
<td>180.28</td>
<td>6.13</td>
<td>165.10–190.60</td>
</tr>
<tr>
<td>Body mass, kg</td>
<td>71.81</td>
<td>5.01</td>
<td>64.80–75.00</td>
</tr>
<tr>
<td>Percent body fat, %</td>
<td>7.87</td>
<td>3.20</td>
<td>4.22–12.77</td>
</tr>
<tr>
<td>VO₂ peak, ml·kg⁻¹·min⁻¹</td>
<td>59.79</td>
<td>5.13</td>
<td>50.30–69.30</td>
</tr>
</tbody>
</table>

VO₂ peak, peak O₂ 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 trials 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-
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 VO2 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-γ, 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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REFERENCES