Carbohydrate supplementation and exercise-induced changes in T-lymphocyte function

Katherine J. Green,1,2 Susan J. Croaker,1 and David G. Rowbottom1
1School of Human Movement Studies, Queensland University of Technology, Kelvin Grove 4057; and 2Cellular Immunology Laboratory, Queensland Institute of Medical Research, Herston 4006, Australia

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Green, Katherine J., Susan J. Croaker, and David G. Rowbottom. Carbohydrate supplementation and exercise-induced changes in T-lymphocyte function. J Appl Physiol 95: 1216–1223, 2003; 10.1152/japplphysiol.00179.2003.—Carbohydrate (CHO) ingestion during exercise has been shown to reduce perturbations in immune cell numbers and function, possibly through a reduction in the cortisol response to exercise. We have previously observed that exercise decreases T-lymphocyte responses to mitogen via an increase in cell death of both CD4 and CD8 T lymphocytes (Green KJ and Rowbottom DG. J Appl Physiol. 95: 57–63, 2003). This study tested the hypothesis that CHO ingestion rather than placebo (Pl) would result in an attenuation of the cortisol response to exercise and a reduction of the exercise-associated alterations in cell death. Six well-trained cyclists completed two exercise trials consisting of 2.5 h of cycling at 85% of individual ventilatory threshold. In a random order, trials were completed under either CHO (6% CHO solution, 3.2 g CHO/kg body wt total) or Pl conditions. Blood samples were collected before exercise, midexercise (after 60 min of exercise), immediately after exercise, and after 60 min of recovery. T-lymphocyte responses to mitogen were determined by using carboxyfluorescein diacetate succinimidyl ester fluorescent cell division tracking and expansion rates, and cell death rates were calculated for each sample as well as mitosis rates for each cell generation. Cellular expansion of T lymphocytes was decreased after exercise in Pl only. The reduction in cellular expansion was related to an increase in cell death of both CD4 and CD8 cells in culture rather than a decrease in the ability of cells to undergo mitosis. CHO ingestion compared with Pl was associated with no reductions in cellular expansion or increases in cell death. CHO ingestion during exercise acted to reduce the impairment of T-lymphocyte function by decreasing cell death within mitogen-stimulated cell cultures; however, the mechanism of action appears to be independent of cortisol.

fluorescent-cell tracking; mitogen-induced proliferation; long-duration exercise; immune function; glucose; cortisol

THERE IS BOTH SCIENTIFIC and anecdotal evidence that athletes suffer from an increased number of infections after a single intensive competitive event (12, 17, 26, 35). T lymphocytes play a central role in mounting and regulating the response to both intra- and extracellular pathogens. Several studies have found that mitogen-induced proliferation of T lymphocytes is decreased after acute exercise (8, 18, 38, 41). It is well known that intensive exercise is also associated with alterations in several immunoregulatory hormones (27, 32). Although the mechanisms are unclear, it has been reported that carbohydrate (CHO) feeding during exercise results in reduced perturbations to both immunoregulatory hormones and immune indexes, including cell numbers and T-lymphocyte function (28).

Although the ergogenic effect of CHO feeding during exercise has been acknowledged for some time, recent investigation has examined the potential role for CHO in maintaining immune function during and after intense exercise. Several studies (13, 24, 28) have identified a beneficial effect of CHO supplementation on immune function during and after exercise. Henson et al. (13) found that CHO ingestion, compared with placebo (Pl), prevented a decline in phytohemagglutinin (PHA)-induced T-lymphocyte proliferation after 2.5 h of treadmill running. There remains, however, a need to clearly identify the mechanism by which CHO may help maintain immune function during acute exercise.

One potential explanation for the role of CHO supplementation in attenuating the reduction in T-lymphocyte function after acute intensive exercise may be the stress hormone cortisol. There is a reported reduction in T-lymphocyte function (13, 25, 33) and an elevation in cortisol (7, 19, 43) after long-duration intense exercise, and the immunosuppressive effects of cortisol are widely recognized (9, 34). Several studies (13, 29) have found a relationship between postexercise cortisol concentration and lymphocyte function, whereas other studies (22) have not. Furthermore, CHO ingestion has been reported to attenuate the usual increase in cortisol concentration with long-duration intense exercise (13, 22).

The potential mechanism by which cortisol influences T-lymphocyte function after exercise is unknown. Cortisol may directly alter lymphocyte function via interaction with receptors on the surface of T lymphocytes (1), or it may indirectly alter proliferative function by inducing changes in composition of the cellular pool (16, 38). Although CHO ingestion has been shown to reduce cortisol release into the bloodstream and diminish changes to immune function (28),
the mechanism behind CHO-induced changes in immune cell function and a causal relationship with cortisol has not been clearly demonstrated. Furthermore, understanding of the connection between exercise, cortisol, and T-lymphocyte function has been hindered by the use of techniques to assess lymphocyte responses to mitogen that do not provide an accurate account of cell division on a per cell basis.

We have previously shown that using carboxyfluorescein succinimidyl ester (CFSE) to quantify cell division can provide unique insights into cell function during and after exercise (11). In addition, we have shown that acute intensive exercise is associated with increased death of both CD4 and CD8 cells in culture in response to PHA (11). There is a strong association between glucocorticoids and T-lymphocyte apoptosis (36, 37) and the potential for CHO ingestion to reduce the cortisol response to exercise. This study investigated the effect of CHO ingestion during long-duration intense exercise on the function of the T-lymphocyte subsets CD4 and CD8 cells by using the CFSE division method (15). We hypothesized that CHO ingestion rather than Pl would result in an attenuation of the cortisol response to exercise and a reduction of the exercise-associated alterations in cell death.

METHODS

Subjects. Six well-trained male cyclists, who fulfilled the selection criteria of a current 40-km trial time of 60 min or less, were recruited for this study. All subjects completed two identical exercise trials during which they received either a CHO or Pl drink, in a randomized order. Subjects were informed of the purpose and risks of participation in the study before consent was obtained. The University Human Research Ethics Committee of the Queensland University of Technology approved this study.

Maximal incremental exercise test. Subjects completed a continuous incremental cycling test on a computerized cycle ergometer (Lode BV Excalibur, Groningen, The Netherlands) to volitional exhaustion. The incremental protocol started at 0 W and increased by 2 W every 5 s until subjects could not maintain cadence above 80 rpm. Breath-by-breath gas analysis was conducted on expired pulmonary gases throughout the test (Medical Graphics CPX/D mobile cart system, Medical Graphics, St. Paul, MN). Maximal oxygen consumption was defined as the single highest 5-s average value attained during the test coinciding with a respiratory exchange ratio of >1.10. Ventilatory threshold was determined from a graph of the ventilatory (Ve) to oxygen consumption (V O2) ratio plotted against time. Threshold was defined as the inflection point of the Ve/V O2 curve and was identified independently by two researchers (44).

Exercise trials. Subjects completed a 3-day food diary over the 3 days before both exercise trials that was analyzed to determine the amount of habitual CHO consumption. Subjects reported to the laboratory at 6:30 AM, having refrained from exercise for the previous 24 h and having only consumed water since midnight. They were fitted with an indwelling venous cannula, through which blood samples were drawn. After a 15-min rest, a blood sample [6:45 AM (T1)] was taken. Subjects completed a standardized warm-up consisting of 5 min of low-resistance cycling followed by stretching. At 7:00 AM, subjects began 150 min of cycling at a constant power output adjusted to elicit a V O2 equivalent to 85% of ventila-
Flow cytometry. After culture, cell suspensions were labeled with CD3 (allophycocyanin), CD8 (phycoerythrin) and Viaprobe (7-aminomethylcoumarin D) for 15 min in the dark at room temperature. Samples were then washed with 3 ml of PBS and resuspended in 200 μl of paraformaldehyde solution (2%) and placed in Trucount tubes (Becton Dickinson). Surface marker and CFSE analysis was conducted by using a FACScalibur flow cytometer (Becton Dickinson). The same forward- and side-scatter parameters were used for each trial as established for human peripheral leukocytes. Standard gating procedures were used to select mainly lymphocytes and to differentiate between labeled and unlabeled cells. Viable lymphocytes were identified by using Viaprobe staining, and forward- and side-scatter parameters. Fluorescent staining was used to further characterize T lymphocytes (CD3) and lymphocyte subsets CD3⁺CD8⁻ and CD3⁺CD8⁺. Data were analyzed by using Flow Jo version 3.3 software (Treestar, San Carlos, CA).

Assessment of lymphocyte expansion. The absolute number of cells in each sample was calculated by determining the ratio of gated lymphocytes or lymphoblasts to gated Trucount beads, multiplied by the known number of beads in the Trucount tube. The increase in the number of viable cells in culture was calculated from the absolute number of cells in the PHA-stimulated sample, relative to the absolute number in the unstimulated sample. This was termed lymphocyte expansion and was completed separately on CD3⁺CD4⁻ and CD3⁺CD8⁻ lymphocyte populations. An expansion value of 1.0 or more indicated that there were a greater number of cells in the PHA-stimulated sample than in the unstimulated sample after 72 h of culture.

Estimation of lymphocyte mitosis and cell death. As CFSE fluorescence intensity halves with mitotic division, each generation of proliferated lymphocytes (G1, G2, etc.) appears as a distinct peak on a CFSE histogram plot (Fig. 1). With the use of separate histograms for CD3⁺CD8⁻ and CD3⁺CD8⁺ lymphocyte populations, and standard histogram gating procedures, the number of cells in each generation was determined for each cell type.

The decrease in parent (G0) cells from unstimulated to PHA-stimulated samples can be attributed to cells undergoing mitosis or cell death. If it is assumed that no cell death occurs, all missing G0 cells must be assumed to have undergone mitosis and would be expected to generate two G1 cells. The same assumption can be made for each generation. There was considerable overestimation of cell numbers in the final generation if the assumption of no cell death was used. Iterative processes were then used to change the assumed cell death rate until all missing cells could be accounted for by mitosis or cell death. The mitosis rate for each generation was then calculated as the percentage of cells estimated to have undergone mitosis. The cell death rate was the percentage of cells estimated to have undergone cell death (apoptosis).

Statistical analysis. All data were expressed as means ± SE throughout and in all graphical presentations. In the case of glucose, cortisol, expansion, and cell death rates, a two-way ANOVA design, with repeated measures on both factors, was conducted. Main effects of time (T1–T4 sample points) and supplement (CHO and Pl) were fitted, as well as the interaction between time and supplement. For mitosis rates, the factors of time and supplement were considered in each generation (G0–G3) separately. To aid interpretation of significant interactions, a one-way ANOVA with main effect of time was carried out for CHO and Pl data separately. Where significant main effects or interactions were observed, Scheffe’s post hoc analysis was carried out. Pearson’s product-moment correlation analysis was carried out between cortisol, glucose, cell numbers, and cell death rates for each variable individual time points as well as averaged data. Statistical significance level was set at \( P < 0.05 \); Cohen’s statistical power was calculated to be >90%.

RESULTS

Subject characteristics. All six subjects (age: 25 ± 5 yr; height: 1.82 ± 0.06 m; weight: 74 ± 8 kg) participated in both exercise trials. The habitual total energy intake of the subjects was 12,590 ± 2,855 kJ/day with CHO consisting of 63.5 ± 6.5% of the total energy intake. The amount of habitual CHO ingestion per kilogram of body weight was 6.825 ± 1.37 g/day and was not significantly different between trials.

Exercise trials. During the incremental maximal test, the mean \( V_{\text{O}2} \) peak value was 56 ± 2 ml·kg⁻¹·min⁻¹ and mean maximum heart rate was 181 ± 8 beats/min. Both exercise trials were completed at the power output identified in the incremental maximal test to be 85% of individual anaerobic threshold (228 ± 25 W), equivalent to 67 ± 2% of maximal \( V_{\text{O}2} \). There was no difference between the mean heart rate during the CHO and Pl trials (145 ± 5 beats/min). One subject failed to complete the 2.5 h ride in the Pl trial because of hypoglycemia and stopped after 2 h of cycling; all data points for this subject were included in the analysis. The total volume of fluid consumed during each trial was 4.0 ± 0.46 liters and was identical between trials for each subject. The total amount of CHO consumed during the CHO trial was 240 ± 28 g.

Glucose and cortisol. There was a significant time × supplement interaction for both glucose and cortisol.
Glucose concentration was significantly higher during recovery (T4) in the CHO trial compared with the Pl trial (Fig. 2). Cortisol concentration was significantly lower immediately postexercise (T3) and in recovery (T4) in the CHO trial compared with the Pl trial. Glucose concentration was significantly higher during exercise (T2) and immediately after exercise (T3) 2.5 h of cycling, and after 1 h of recovery (T4) under carbohydrate (CHO; ○) and placebo (Pl; ●) conditions. *Significant difference between Pl and CHO (P < 0.05).

**Circulating leukocyte numbers.** There were significant time × supplement interactions for the number of circulating WBC and polymorphonuclear neutrophils. Immediately after exercise (T3), the numbers of WBC and polymorphonuclear neutrophils were significantly lower in the CHO trial compared with the Pl trial (Table 1). The numbers of WBC, polymorphonuclear neutrophils, and monocytes were all significantly lower during recovery (T4) in the CHO compared with the Pl trial. Although there was no time × supplement interaction for the number of lymphocytes or monocytes, significant main effects of time were observed in both the CHO and Pl trials. Compared with preexercise (T1), the number of lymphocytes in circulation was significantly lower during recovery (T4) in both trials. Similarly, there was no significant time × supplement interaction for the number of circulating CD3, CD4, or CD8 cells (Table 1). There was a significant main effect of time in both the CHO and Pl trials. The numbers of CD3, CD4, and CD8 cells were significantly higher during exercise (T2), and the numbers of CD3 and CD4 cells were significantly lower during recovery (T4) than the preexercise sample (T1) (time main effects not indicated in table).

**Lymphocyte expansion.** There was a significant time × supplement interaction for CD4 lymphocyte expansion. For CD8 cells, the interaction approached significance (P = 0.057). There was a main effect of time in Pl only; compared with preexercise (T1), lymphocyte expansion of both CD4 and CD8 cells was significantly lower during exercise (T2). For CD4 cells, lymphocyte expansion was also significantly reduced immediately postexercise (T3) in Pl. Although the CHO trial followed the same pattern of change in cellular expansion, there were no significant changes from baseline in either cell type. In Pl, cellular expansion decreased between T1 and T2 from 1.32 ± 0.17 to 0.94 ± 0.25 for CD4 cells and from 1.97 ± 0.38 to 1.29 ± 0.29 for CD8 cells. At all time points, expansion of the CD8 cell population was larger than that of CD4 cells (Fig. 4).

**Lymphocyte mitosis.** There was no significant interaction between time and supplement for lymphocyte mitosis in G0–G2 in either CD4 or CD8 cells, such that the percentage of CD4 or CD8 cells undergoing mitosis in each generation was not significantly different between CHO and Pl trials. The mitosis rate of CD4 cells in G0 was significantly decreased during exercise in both CHO and Pl trials. However, in all other generations for both cell types, there was no effect of time or condition. At all time points, the mitosis rates of CD8 cells were significantly higher compared with CD4 cells, and mitosis rates of each successive cell generation declined significantly (Fig. 5).

**Lymphocyte death.** There was a significant time × supplement interaction for cell death rates of CD4 cells only. There was a significant main effect of time in the Pl trial only for both CD4 and CD8 cells; compared with preexercise (T1), the percentage of apoptotic CD4 and CD8 cells in cell culture was significantly increased during and immediately after exercise (T2 and T3). For CD8 cells, there was also a main effect of supplement because cell death rates were higher in Pl compared with CHO trials. In CHO trials, there was no significant change from baseline in the amount of cell death in cultures during exercise or recovery for either cell type. Cell death increased in Pl trials between T1 and T2 from 12 ± 6 to 28 ± 8% in CD4 cells and from 4 ± 6 to 24 ± 7% for CD8 cells. At all time points, CD4 cells exhibited higher cell death rates than CD8 cells (Fig. 6).

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**Fig. 2.** Changes in the serum glucose concentration before (T1), during (T2), and immediately after (T3) 2.5 h of cycling, and after 1 h of recovery (T4) under carbohydrate (CHO; ○) and placebo (Pl; ●) conditions. *Significant difference between Pl and CHO (P < 0.05).

**Fig. 3.** Changes in serum cortisol concentration before (T1), during (T2), and immediately after (T3) 2.5 h of cycling, and after 1 h of recovery (T4) under carbohydrate (CHO; ○) and placebo (Pl; ●) conditions. *Significant difference between Pl and CHO (P < 0.05).
Correlation analysis. In Pl trials, the number of WBCs at T3 (immediately postexercise) correlated significantly with the cortisol concentration at T2 (1 h of exercise) \((r = 0.93)\). There were no significant correlations between the numbers of WBCs, lymphocytes, glucose, cortisol, cell death, and CD3, CD4, or CD8 cells at any time point in either condition.

### DISCUSSION

The present study investigated the effect of CHO ingestion during long-duration intense exercise on the mitogen responsiveness of T lymphocytes. Several earlier reports (8, 18, 38, 41) have found that mitogen-induced proliferation of T lymphocytes is decreased after acute exercise. It has been reported that CHO feeding during long-duration intense exercise results in reduced perturbations to both immunoregulatory hormones and immune indexes, including cell numbers and T-lymphocyte function (28). It is widely reported that exercise stimulates the hypothalamic pituitary adrenal axis and results in elevated blood cortisol levels (20). In addition to exercise stimuli, cortisol levels are heightened in response to a decrease in blood glucose levels (28). CHO ingestion has been shown to improve glucose availability (3, 4, 10, 42). It has therefore been suggested that exogenous CHO feeding during exercise may influence the immune response to exercise by maintaining blood glucose levels and thereby reducing cortisol (2, 13, 21). We have previously noted that exercise-associated reductions in mitogen responsiveness of T lymphocytes are related to an increase in cell death in culture rather than to a decline in the propensity of cells to divide (11). Given the strong relationship between apoptosis and glucocorticoids (39), one aim of this study was to investigate the potential role of cortisol in exercise-induced cell death.

This study confirmed previous reports that CHO compared with Pl ingestion is associated with higher blood glucose concentrations, reduced cortisol concentration, less perturbation of circulating leukocytes numbers, and an attenuation of exercise-induced decrease in T-cell proliferative function (13). The present study also supports our previous findings that the
exercise-associated impairment of cellular expansion is due to an increase in cell death rather than to a decrease in mitosis (11). Furthermore, these data extend the understanding of how CHO ingestion acts to maintain T-lymphocyte function to suggest that CHO ingestion during exercise acts to prevent cell death (Fig. 6). CHO ingestion during exercise (compared with Pl) prevented any reduction in the expansion of the cell population by avoiding any exercise-associated increase in the death of cells in culture (Fig. 4).

We have previously proposed two possible explanations as to why cell death rates are increased with exercise (11). In this study, we have further explored these possibilities to examine the mechanism by which CHO ingestion is capable of preventing exercise-induced cell death. High cell death rates occurred during culture of cells taken during and immediately after exercise; these time points coincide with the greatest number of circulating lymphocytes. Previous studies have identified that CHO ingestion is connected with significant reductions in the extent of leukocytosis during exercise (30, 31). CHO ingestion acts to moderate fluctuations in lymphocyte numbers, in particular, CHO compared with Pl is linked to a reduction in the concentrations of lymphocytes immediately postexer-
exercise and an increase after 3 h of recovery (13). Because exercise-induced lymphocytosis is largely due to an influx of immature cells and cells from marginized pools, the composition of the lymphocyte pool is considerably different during and immediately after exercise. Increased cell death rates at these time points may potentially have been explained by an increased susceptibility to apoptosis of the new cells in the circulation. However, our present data do not support this speculation. CHO ingestion significantly reduced cell death rates, particularly at the 1-h exercise time point (T2), despite no difference in lymphocyte numbers between PI and CHO trials. We also found no significant correlation between the numbers of cells in circulation and apoptosis rates.

It is widely recognized that glucocorticoids are capable of inducing apoptosis of lymphocytes (14, 39); therefore, an alternative explanation for the exercise-associated increase in cell death may be due to alterations in immunomodulators such as cortisol. It has previously been shown that, during and after exercise, the time course of suppression of lymphocyte proliferation is inversely related to that of plasma cortisol (33). One proposed mechanism by which CHO ingestion during exercise reduces changes in immune cell numbers and function is through blunting the cortisol response to exercise (28). However, in the present study, we found no relationship between cortisol concentrations and changes in T-lymphocyte numbers or function. We observed the highest rates of cell death in samples taken during exercise, an effect that was significantly blunted by CHO ingestion, despite no effect of CHO ingestion on serum cortisol concentration during exercise. CHO supplementation only manifested a reduction in cortisol concentration postexercise and during recovery (Fig. 3). The disparity between the time points of attenuating effects of CHO would indicate that CHO ingestion acted through some mechanism other than cortisol to reduce cell death associated with exercise.

Because data from the present study do not directly implicate cortisol in the mediation of lymphocyte death after exercise, the reduction in apoptosis with CHO ingestion may be related to other factors, such as glucose availability or adrenaline concentration. Glucose is well established as an essential nutrient for optimal immune cell function during exercise (2), and CHO ingestion has been shown to improve its availability (3, 4, 10, 42). Similarly, epinephrine has been shown to be elevated during exercise and to have immunosuppressive effects (5, 6, 40). There are also reports that epinephrine concentrations are suppressed by CHO ingestion during exercise (21, 23). Although we did not measure epinephrine in this study, it is worthy of investigation in future studies of the mechanism behind exercise changes in apoptosis of T lymphocytes.

This study confirms our previous finding that reductions in the mitogen response of T lymphocytes after exercise are related to an increase in cell death of both CD4 and CD8 cells in culture (11). We have also identified that CHO ingestion during exercise acts to reduce the impairment of T-lymphocyte function by decreasing death in cell cultures. In addition, our results indicate that mechanisms independent of changes in cell numbers and cortisol may be responsible for exercise-induced apoptosis. We suggest that other immunomodulatory factors, possibly the direct role of glucose availability or epinephrine, may provide a more consistent hypothesis regarding exercise-associated changes of in vitro immune cell function. Further research is also needed to establish the connection between in vitro cell death and susceptibility to infection in athlete groups.

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