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# THE EFFECT OF CARBOHYDRATE SUPPLEMENTATION DURING THE RECOVERY INTERVAL ON IMMUNOENDOCRINE RESPONSES TO A REPEATED BOUT OF PROLONGED CYCLING

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## Abstract

**Aim of the study:** The purpose of this study was to examine the effect of carbohydrate feeding during the recovery interval (REC) separating two 90-min cycling bouts (EX1 started at 09:00 and EX2 started at 13:30) at 60% $\dot{V}O_2$max on leukocyte redistribution, neutrophil degranulation response to lipopolysaccharide (LPS) and plasma IL-6 and stress hormone responses to EX2.

**Methods:** Subjects (n = 8) consumed a 10% w/v glucose or placebo beverage (22 mL·kg$^{-1}$ body mass) during the first hour of REC in two experimental trials, which were completed in a counterbalanced order and separated by at least 4 days. Venous blood samples were taken 5 min before exercise and immediately post-exercise for both trials.

**Results:** The main findings were that ingestion of carbohydrate compared with placebo during REC blunted the increase in the blood leukocyte count, plasma ACTH and cortisol concentrations, and total neutrophil degranulation response to LPS during EX2. Furthermore, EX2 evoked significantly higher circulating numbers of leukocytes, neutrophils, lymphocytes and monocytes, higher plasma concentrations of stress hormones, and total LPS-stimulated elastase release.

**Conclusions:** These findings suggest that compared with placebo, carbohydrate ingestion during REC between two bouts of prolonged exercise attenuates hypothalamic-pituitary-adrenal axis activation; however, this has only a limited effect on perturbations of circulating leukocyte subsets and neutrophil function during the second exercise bout. A second prolonged exercise bout causes a greater disturbance in immunoendocrine responses than the first exercise bout on the same day and this effect is not substantially attenuated by CHO supplementation during the recovery interval.

*Key words: leukocyte redistribution, neutrophil degranulation, IL-6, stress hormones*

## Introduction

Leukocyte trafficking between the circulation and other compartments is crucial for pathogen surveillance (7). Exercise has been shown to induce a significant but reversible mobilisation of leukocyte subsets into the circulation from marginated pools and the bone marrow (16). This effect is associated with rises in plasma levels of stress hormones (1) and cytokines including interleukin (IL)-6 (28). The latter is mainly produced by contracting skeletal

muscles (39) and acts in a hormone-like fashion to maintain plasma glucose homeostasis and stimulate lipolysis during exercise (11, 15).

Neutrophils (50-60% of the blood leukocytes) act as the first line of defence against pathogens and are regarded as one of the best phagocytes in innate immunity (36). Following activation, neutrophils destroy invading pathogens via two processes: oxygen-independent (release of proteases) and oxygen-dependent (release of reactive oxygen species, ROS) mechanisms (12, 19). Previous studies have demonstrated that endurance training temporarily blunts the activities of neutrophil phagocytosis (4), degranulation (5) and oxidative burst (13, 31). However, no study to date has reported the effects of two successive bouts of prolonged exercise on neutrophil degranulation or the possible modifying effect of carbohydrate (CHO) ingestion during the recovery interval.

Numerous studies have been done to examine the influence of an acute single exercise bout on the immune system and the results indicate that after intensive prolonged exercise, such as a marathon race, cellular immunity is depressed for several hours (23). During this so-called "open window" period, pathogens may invade and cause infections in stressed athletes (27). The training regimens of many athletes involve several bouts of exercise in a day. Although a few studies have examined the effects of daily repeated exercise bouts on immunoendocrine responses (33, 34), no study has investigated the influence of CHO ingestion during repeated bouts of exercise on immunoendocrine responses.

During stressful periods of training involving multiple exercises with short recovery intervals, it is particularly important for athletes to maintain immunocompetence (14) and rapidly restore glycogen (24) during recovery if they are to maintain training quality in subsequent bouts of exercise. It has been suggested that inadequate nutrition may result in glycogen depletion and subsequent elevation of stress hormones with impairment of immune cell function during prolonged exercise, leading to an increased incidence of infection (41). The ingestion of CHO compared with placebo (PLA) in drinks consumed during exercise appears to better maintain plasma glucose concentration, improve endurance exercise performance, and attenuate the elevation of plasma stress hormones and perturbation of circulating counts of total leukocytes and leukocyte subsets (17).

Price *et al.* (29) reported that the recovery of glycogen after glycogen depleting exercise is biphasic: an initial rapid phase (insulin-independent) of glycogen synthesis ($27 \pm$ mmol·L$^{-1}$·h$^{-1}$, determined by $^{13}$C-nuclear magnetic resonance spectroscopy) lasting 30 - 60 min is followed by a slower (insulin-dependent) phase ($2.9 \pm 0.8$ mmol·L$^{-1}$·h$^{-1}$). If CHO is not provided during recovery, glucose availability will be the limiting factor for glycogen synthesis since gluconeogenesis would not be able support the maximal rate of glycogen synthesis (35). In the present study subjects were provided with 2.2 g CHO · kg$^{-1}$ body mass during the first hour of the recovery period, which was estimated to be equivalent to the amount of CHO oxidised during the first exercise bout and is comparable with the recommendation of Ivy *et al.* (18), who suggested that the optimal strategy to restore glycogen is to consume ~1.5 g CHO · kg$^{-1}$ body mass immediately after exercise and similar amounts at 2 h intervals thereafter.

Therefore, the first aim of the present study was to examine the effect of carbohydrate supplementation during the recovery interval on leukocyte mobilisation,

neutrophil degranulation response to lipopolysaccharide (LPS), plasma IL-6 and stress hormone responses to a second bout of prolonged exercise. The second aim was to compare the magnitude of the immunoendocrine response between the first and the second exercise bout. We hypothesised that carbohydrate ingestion may be beneficial to blunt the perturbation of immunoendocrine responses during a subsequent exercise bout and that the second exercise bout would evoke greater immunoendocrine responses compared with the first bout.

## Methods

### *Subjects*

Eight male volunteers (age 29.8 ± 1.6 years, height 175 ± 2 cm, body mass 75.4 ± 2.6 kg, $\dot{V}O_2$max 45.5 ± 2.2 mL·kg$^{-1}$·min$^{-1}$; means ± S.E.M.), who were recreationally active and familiar with cycling, participated in the study. After receiving written information about this study and passing a Health Questionnaire screen, subjects signed an informed consent. Subjects were requested to complete the dietary record sheet on the day prior to Trial 1 and then repeated it again before Trial 2. Subjects were also asked not to perform any strenuous exercise or consume alcohol or medication for 2 days before each trial. The protocol was approved by the Ethics Committee of Loughborough University before the study began.

### *Preliminary Measurements*

Maximal oxygen uptake $\dot{V}O_2$max was estimated by means of a continuous incremental exercise test on a cycle ergometer (Monark 874E, Monark Exercise AB, Sweden) to volitional exhaustion. Subjects began cycling at 70 W with increments of 35 W every 3 min. The cadence was maintained at 70 rev·min$^{-1}$ and heart rate was monitored using radiotelemetry. During the third minute of each work rate increment, expired gas was collected in Douglas bags. An oxygen/carbon dioxide analyzer (Servomax 1400B, Crowborough, UK) was used along with a dry gas meter (Harvard Apparatus, Edenbridge, UK) for determination of $\dot{V}O_2$ and $\dot{V}CO_2$. From the $\dot{V}O_2$-work rate relationship, the work rate equivalent to 60% $\dot{V}O_2$max was interpolated.

### *Experimental Procedures*

The subjects completed two trials in a counterbalanced order, each separated by at least 4 days. Subjects arrived at the laboratory at 08:30 after fasting from 23:00 the previous day and were asked to empty the bladder before body mass was recorded. Subjects then performed two bouts of 90 min cycling (EX1 started at 09:00 and EX2 started at 13:30) at 60% $\dot{V}O_2$max at 70 rev·min$^{-1}$ on the same ergometer used to determine $\dot{V}O_2$max. Subjects consumed a lemon flavoured 10% w/v CHO (glucose) beverage or artificially sweetened placebo (22 mL·kg$^{-1}$ body mass) during the first hour of the recovery period (i.e. 10:30-11:30). The amount of CHO ingested (~165 g) was approximately equivalent to the amount of CHO oxidised during EX1 based on measurements of respiratory gas exchange. Heart rate was recorded continuously during exercise by radiotelemetry. Ratings of perceived exertion (RPE) were obtained at 15-min intervals. Venous blood samples were taken 5 min before exercise and immediately post-exercise. Water ingestion was allowed *ad libitum* during the exercise bouts. The laboratory temperature and relative humidity were 23.9 ± 0.1 $^{o}$C and 31 ± 1%, respectively.

### *Blood Collection and Analysis*

Venous blood samples were taken from an antecubital vein by venepuncture, and were collected into three Vacutainer tubes

(Becton Dickinson, Oxford, UK). Blood samples in two $K_3$EDTA vacutainers (4 mL) were used for haematological analysis including haemoglobin, haematocrit, and total and differential leukocyte counts using an automated haematology analyzer ($A^C$•$T^{TM}$ 5diff analyser, Beckman Coulter, UK) and to determine changes in the plasma concentrations of stress hormones and interleukin-6. Plasma volume changes were calculated according to Dill and Costill (9). From blood taken into a lithium heparin vacutainer (7 mL), 1 mL was immediately added to an eppendorf tube (1.5 mL capacity) containing 50 $\mu$L of 10 mg·mL$^{-1}$ bacterial lipopolysaccharide (LPS) solution (Stimulant, Sigma, Poole, UK). Blood and LPS were mixed by gentle inversion and then incubated for 1 h at 37˚C, with gentle mixing every 20 min. After incubation, the mixture was centrifuged for 2 min at 15000 *g*. The supernatant was immediately stored at –80˚C prior to analysis of elastase concentration. The amount of elastase released per neutrophil in response to LPS stimulation was calculated according to Robson *et al*. (32).

The remaining $K_3$EDTA and heparinized whole blood was spun at 1500 *g* for 10 min in a refrigerated centrifuge at 4˚C within 10 min of sampling. The plasma obtained was immediately stored at –80˚C prior to analysis. Plasma aliquots were analyzed to determine the concentration of glucose (GOD-PAP method, Randox, Ireland) using an automatic photometric analyzer (Cobas-Mira plus, Roche). Human growth hormone (GH), cortisol (both DRG Instruments GmbH, Germany), adrenaline (IBL GmbH, Hamburg), interleukin-6 (IL-6) (high sensitivity kit produced by Diaclone Research, France. According to the manufacturer, the minimum detectable sample concentration is less than 0.8 ng·L$^{-1}$ and the intra-assay and inter-assay CV% are 5.5 and 1.4, respectively, at the concentration of 2.7 ng · L$^{-1}$) and elastase (Merck, Lutterworth, UK) were determined using enzyme-linked immunosorbant assay (ELISA) kits. The intra-assay coefficient of variation based on duplicate analyses in our laboratory was 1.3%, 2.4%, 6.9%, 12.7%, 1.6%, and 3.9% for glucose, GH, cortisol, adrenaline, IL-6, and elastase, respectively.

**Statistical analysis**

All results are presented as mean values and standard errors of the mean (± SEM). Data were checked for normality, homogeneity of variance and sphericity before statistical analysis, and where appropriate the Huynh-Feldt method was applied for adjustment of degrees of freedom for the *F*-tests. Comparisons of immunoendocrine responses during EX2 were analyzed using a two-factor (2 trials × 2 time points) repeated measures ANOVA with *post hoc t*-tests. Physiological variables, RPE and comparisons of immunoendocrine responses between post-EX1 and post-EX2 were examined using paired *t*-tests. *P*, *t*, and adjusted *F* values are presented and statistical significance was accepted at $P < 0.05$.

**Results**

***Physiological Variables and Ratings of Perceived Exertion (RPE)***

There was no difference between trials in exercise intensity (%$\dot{V}O_2$max), heart rate, RPE, body mass loss, water intake and the percentage change in plasma volume (Table 1). However, the RPE response in EX2 was significantly higher compared with EX1 ($t = 2.5$, $P = 0.048$ for CHO and $t = 5.3$, $P = 0.002$ for PLA). The exercise intensity and the percentage of plasma volume change in EX2 were significantly higher compared with EX1 in the PLA trial ($t = 3.8$, $P = 0.007$) and in the CHO trial ($t = 3.0$, $P = 0.019$), respectively.

Table 1. *The exercise intensity and its effect on heart rate (HR), ratings of perceived exertion (RPE), body mass loss, water intake, and percentage change in plasma volume*

| | CHO | | PLA | |
|---|---|---|---|---|
| | EX1 | EX2 | EX1 | EX2 |
| % $\dot{V}O_2$max | 59.6 (1.3) | 62.0 (1.3) | 60.2 (1.2) | 63.1 (1.0)** |
| HR (beats·min$^{-1}$) [a] | 140 (3) | 146 (3) | 142 (4) | 148 (3) |
| RPE [a] | 13.1 (0.4) | 15.0 (0.5)* | 13.4 (0.3) | 15.1 (0.2)** |
| Body mass loss (kg) [b] | 1.19 (0.15) | 1.23 (0.19) | 1.26 (0.18) | 1.22 (0.13) |
| Water intake (mL) | 686 (127) | 833 (99) | 800 (129) | 808 (51) |
| Plasma volume change (%) [c] | - 4.0 (0.5) | - 3.0 (0.4)* | - 3.9 (0.7) | - 3.3 (0.5) |

Values are mean (± SEM, n = 8). Significantly different from EX1 (*$P$ < 0.05, **$P$ < 0.01). [a] Measurements made in last 15 min of exercise. [b] After correction for water intake. [c] Immediately post-EX compared with pre-EX.

### *Leukocyte counts*

There was a significant main effect of trial for the blood counts of leukocytes ($F_{1, 7}$ = 6.3, $P$ = 0.040, Figure 1A) and lymphocytes ($F_{1, 7}$ = 6.0, $P$ = 0.045, Figure 1C), which showed higher values in PLA compared with CHO. There was also a significant main effect of time for the blood leukocyte ($F_{1, 7}$ = 39.8, $P$ < 0.001), lymphocyte ($F_{1, 7}$ = 105.7, $P$ < 0.001) and monocyte ($F_{1, 7}$ = 34.2, $P$ = 0.001, Figure 1D) counts during EX2, with a higher va-

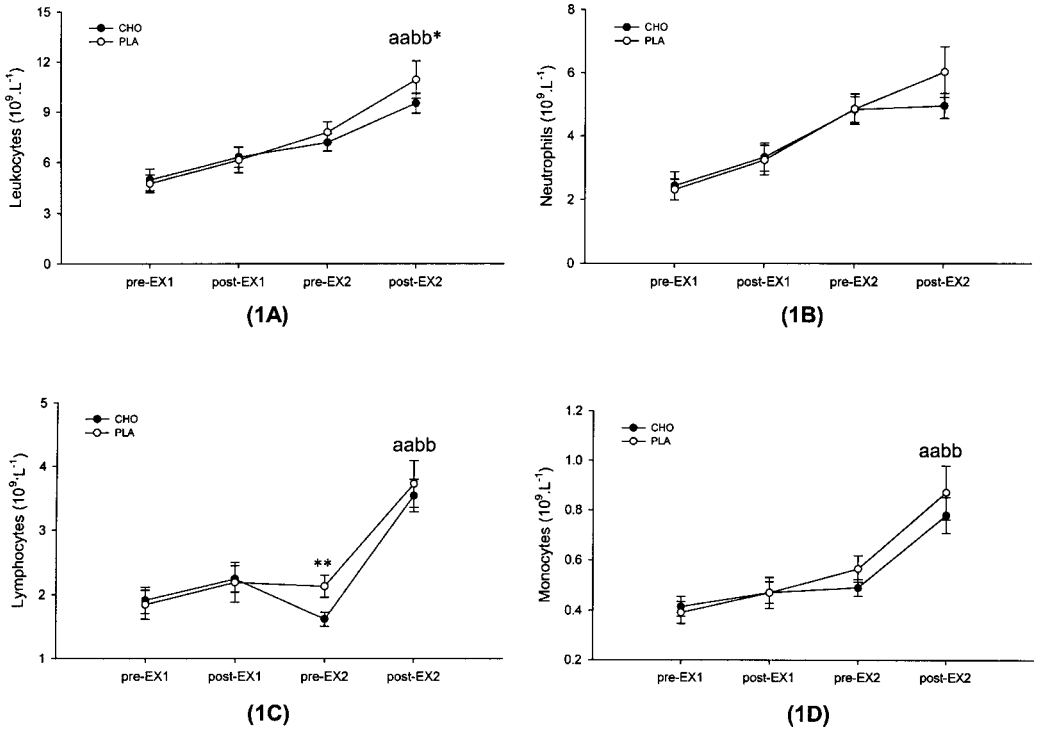


Figure 1. *Changes in circulating counts of total leukocytes (1A), neutrophils (1B), lymphocytes (1C), and monocytes (1D). Values are means ± SEM (n = 8). Significantly different from pre-EX2 in CHO ($^{aa}$ $P$ < 0.01) and PLA ($^{bb}$ $P$ < 0.01); significantly different between trials ($^{*}$ $P$ < 0.05, $^{**}$ $P$ < 0.01).*

lue at post-EX2 in both trials. Although there was no main effect of trial or time for circulating neutrophils, a significant interaction between trial and time was observed during EX2 ($F_{1,7} = 8.3$, $P = 0.024$, Figure 1B).

EX2 induced a greater leukocytosis ($t = 9.6$, $P < 0.001$ for CHO and $t = 7.7$, $P < 0.001$ for PLA), neutrophilia ($t = 5.9$, $P = 0.001$ for CHO and $t = 5.6$, $P = 0.001$ for PLA), lymphocytosis ($t = 6.4$, $P < 0.001$ for CHO and $t = 11.2$, $P < 0.001$ for PLA) and monocytosis ($t = 6.8$, $P < 0.001$ for CHO and $t = 5.7$, $P = 0.001$ for PLA) compared with EX1.

### *Stress Hormones*

There was a significant main effect of trial for plasma concentrations of ACTH ($F_{1,7} = 8.0$, $P = 0.026$, Figure 2B) and cortisol ($F_{1,7} = 8.0$, $P = 0.026$, Figure 2C), which showed higher values in PLA compared with CHO. There was also a significant main effect of time for plasma concentrations of ACTH ($F_{1,7} = 14.3$, $P = 0.007$), cortisol ($F_{1,7} = 33.9$, $P = 0.001$) and GH ($F_{1,7} = 14.1$, $P = 0.007$, Figure 2D), with higher levels at post-EX2 than pre-EX2 in both CHO and PLA trials.

EX2 induced a larger rise in plasma adrenaline concentration ($t = 3.8$, $P = 0.007$ for CHO and $t = 5.0$, $P = 0.002$ for PLA; Figure 2A), ACTH ($t = 2.6$, $P = 0.035$ for CHO and $t = 3.4$, $P = 0.011$ for PLA), cortisol ($t = 2.7$, $P = 0.032$ for CHO and $t = 6.9$, $P < 0.001$ for PLA) and GH ($t = 2.7$, $P = 0.032$ for CHO and $t = 4.3$, $P = 0.003$ for PLA) compared with EX1 at post-exercise.

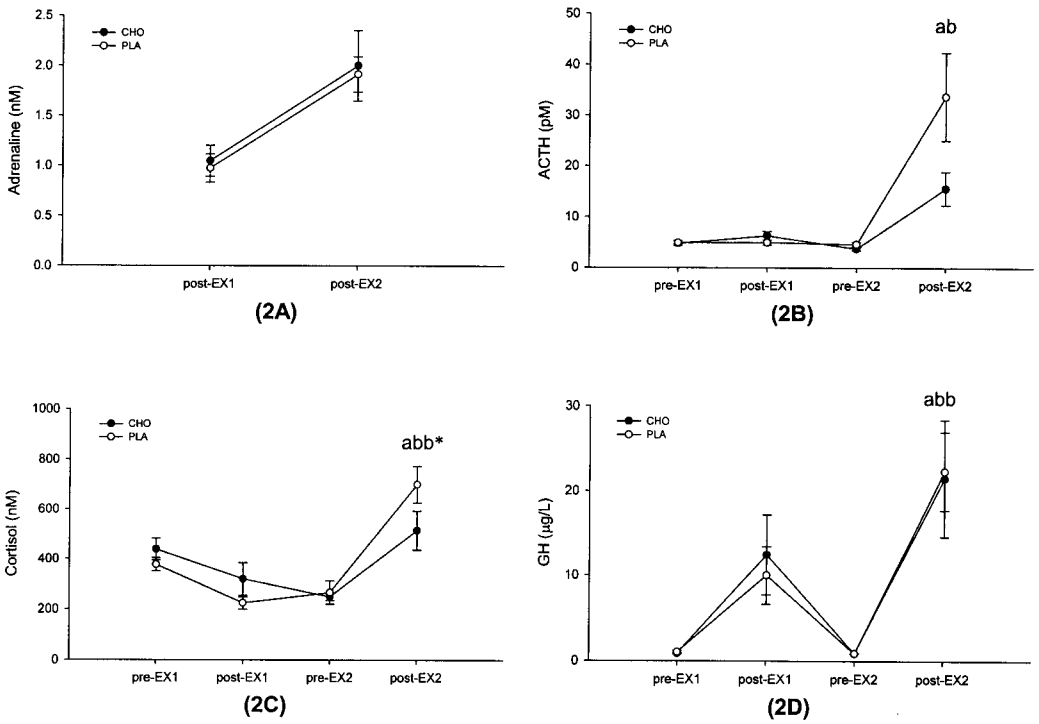


Figure 2. *Changes in plasma concentrations of adrenaline (2A), ACTH (2B), cortisol (2C), and growth hormone (2D). Values are means ± SEM (n = 8). Significantly different from pre-EX2 in CHO ([a] $P < 0.05$) and PLA ([b] $P < 0.05$); significantly different between trials ([*] $P < 0.05$).*

### *Glucose and IL-6*

There was a significant main effect of time for concentrations of plasma glucose ($F_{1,7}$ = 19.2, $P$ = 0.003, Figure 3A) and IL-6 ($F_{1,7}$ = 21.5, $P$ = 0.002, Figure 3B), with lower plasma glucose, but higher plasma IL-6, at post-EX2 compared with pre-EX2 in both trials. EX2 significantly decreased plasma glucose concentrations ($t$ = 4.0, $P$ = 0.005 for CHO and $t$ = 6.3, $P$ < 0.001 for PLA) compared with post-EX1 but only marginally increased plasma IL-6 levels in PLA ($t$ = 3.0, $P$ = 0.019 for PLA and $t$ = 1.5, $P$ = 0.174 for CHO).

### *Neutrophil Degranulation*

There were significant main effects of trial ($F_{1,7}$ = 8.1, $P$ = 0.025) and time ($F_{1,7}$ = 5.7, $P$ = 0.049) and an interaction between trial and time ($F_{1,7}$ = 9.9, $P$ = 0.016) for total LPS-stimulated elastase release, which was higher at post-EX2 in PLA compared with pre-EX2 and post-EX2 in CHO (Figure 4A). There was no difference in LPS-stimulated elastase release per neutrophil in both trials during EX2 (Figure 4B). At post-EX2, the total LPS-stimulated elastase release was significantly higher than post-EX1 in both trials ($t$ = 3.6, $P$ = 0.009 for CHO and $t$ = 4.6, $P$ = 0.002 for PLA). However, there was no difference between post-EX2 and post-EX1 for LPS-stimulated neutrophil degranulation expressed on a per cell basis.

## Discussion

The main findings of the present study were that ingestion of CHO compared with

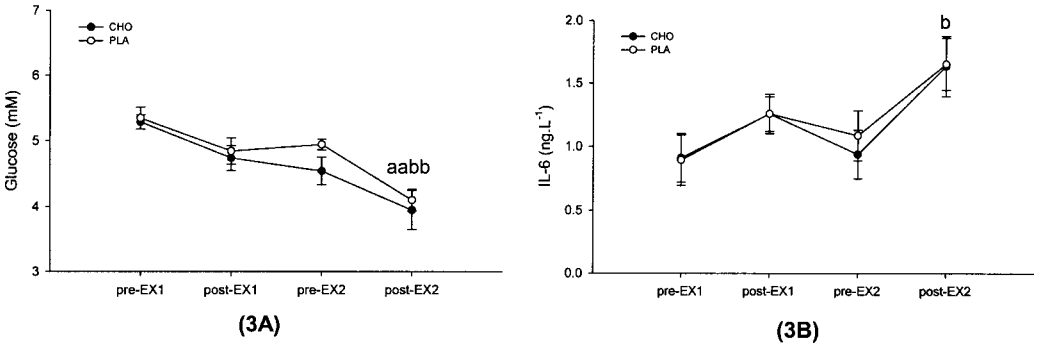


Figure 3. *Changes in plasma concentrations of glucose (3A) and plasma IL-6 (3B). Values are means ± SEM (n = 8). Significantly different from pre-EX2 in CHO ($^{aa}$ P < 0.01) and PLA ($^{b}$ P < 0.05, $^{bb}$ P < 0.01).*

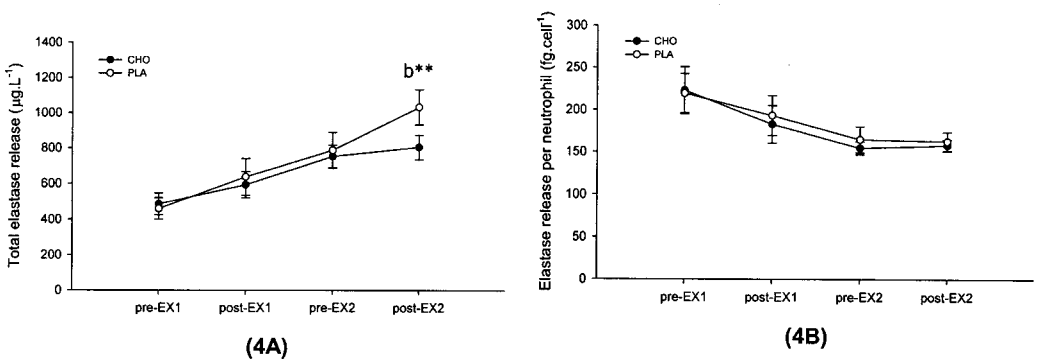


Figure 4. *Changes in total LPS-stimulated elastase release (4A) and LPS-stimulated elastase release per neutrophil (4B). Values are means ± SEM (n = 8). Significantly different from pre-EX2 in PLA ($^{b}$ P < 0.05); significantly different between trials ($^{**}$ P < 0.01).*

PLA during the recovery interval blunted the increase in blood total leukocyte counts, plasma ACTH and cortisol concentrations, and total neutrophil degranulation during EX2 but did not affect the changes in plasma glucose concentration, neutrophil degranulation on per cell basis, circulating counts of neutrophils, lymphocytes and monocytes, and plasma concentrations of adrenaline, GH and IL-6 during EX2. Furthermore, EX2 evoked significantly higher circulating numbers of leukocytes, neutrophils, lymphocytes and monocytes, higher plasma concentrations of adrenaline, ACTH, cortisol, GH, higher total LPS-stimulated elastase release, and lower plasma glucose levels compared with EX1 in both CHO and PLA trials.

CHO supplementation during the first hour of the recovery interval was intended to replace previously depleted hepatic and muscle glycogen stores to enhance the CHO availability during the subsequent 90 min bout of cycling. Although CHO ingestion did not attenuate the decrease of plasma glucose concentration compared with PLA in this study, no hypoglycaemia was found throughout the experimental protocol in either trial. This suggests that endogenous CHO availability was still sufficient in PLA throughout two bouts of 90 min cycling at 60%$\dot{V}O_2$max.

The alterations in plasma ACTH and cortisol, but not adrenaline and GH, during EX2 were blunted by CHO ingestion. Exercise has been recognised as a stressor to physiological homeostasis (37) and disturbance of homeostasis subsequently activates the sympathetic nervous system (SNS) and the hypothalamic-pituitary-adrenal (HPA) axis, resulting in the elevated plasma concentrations of catecholamines and glucocorticoids (10). However, CHO supplementation before and during exercise can blunt HPA activation (25) and the CHO ingestion in the present study was at least partly effective in attenuating HPA activation.

Circulating counts of neutrophils, lymphocytes and monocytes were significant increased after EX2; however, there were no differences between CHO and PLA trials. The result may be mainly attributable to the higher plasma stress hormone responses during EX2. Previous infusion studies have consistently demonstrated that adrenaline mobilises leukocytes from marginated pools into the circulation in a biphasic manner, starting with an initial lymphocytosis within 10 min, peaking at 30 min, and followed by a neutrophilia with relative lymphopenia, peaking at 2 to 4 h (1). Kappel *et al*. (20) also reported a circulating neutrophilia after an intravenous GH injection. Although plasma cortisol also plays a prominent role in the regulation of leukocyte redistribution (6, 8), it does not appear to dominate until after 90 min of exercise (26). Thus, the circulating neutrophilia, lymphocytosis, and monocytosis during both bouts of 90 min cycling is probably mainly attributable to the influence of plasma adrenaline and GH. We also found a profound increase in blood counts of leukocyte subsets and concentrations of plasma stress hormones at post-EX2 compared with post-EX1. This observation agrees with the findings of our recent study (22) and other previous studies (33, 34), which showed that the second exercise bout on the same day evokes more pronounced changes in leukocyte subsets and stress hormones compared with the first bout of identical exercise on the same day.

In the present study, we did not find a significant difference in plasma IL-6 levels at post-EX2 between CHO and PLA. However, this is not surprising because IL-6 is not produced in large amounts by contracting muscle until glycogen is nearly depleted (11). The relative low IL-6 con-

centrations (only about 1.65 ng·$L^{-1}$) at post-EX2 in this study were similar to the results of previous studies (21, 38), which showed the values of IL-6 between 1-3 ng·$L^{-1}$ at similar workloads and may indicate that subjects did not deplete their muscle glycogen after two bouts of 90 min cycling at 60%VO2max. Although IL-6 has been suggested to maintain glucose homeostasis and stimulate lipolysis during exercise (15), the relatively low plasma concentrations of IL-6 in this study may not exert significant metabolic effects because the criterion level for initiating acute metabolic responses may be as high as 25-65 ng·$L^{-1}$ (40).

After EX2, significantly higher levels of total LPS-stimulated elastase release were found in PLA compared with CHO. However, no difference was apparent after dividing by blood neutrophil counts to express the neutrophil degranulation response on a per cell basis. LPS-stimulated elastase release per neutrophil was not significantly decreased until 3 h after EX1 in both trials and there was no further decline during EX2 and no difference between CHO and PLA throughout the experimental protocol. This finding was similar to previous studies of single bouts of prolonged exercise, which have reported a reduced response of neutrophil degranulation to LPS stimulation *in vitro* on per cell basis (32, 42) and found that CHO ingestion did not blunt the decline of LPS-stimulated elastase release per neutrophil during exercise (2, 3). The delayed decline of LPS-stimulated elastase release per neutrophil may be due to the influence of plasma cortisol because cortisol is known to induce delayed neutrophilia with less mature neutrophils entering the circulation from the bone marrow (30). We observed significantly higher counts of circulating neutrophils at pre-EX2 compared with post-EX1, which coincides with the decreased neutrophil degranulation responses to LPS.

In conclusion, the findings of the present study suggest that carbohydrate ingestion during the recovery interval between two bouts of prolonged exercise attenuates HPA activation; however, this has only a limited effect on attenuating the perturbations of circulating leukocyte subsets and neutrophil function during the second exercise bout. A second prolonged exercise bout causes a greater immunoendocrine response than the first exercise bout on the same day but this is not substantially influenced by carbohydrate supplementation during the recovery interval.

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