Carbohydrate Ingestion Influences Skeletal Muscle Cytokine mRNA And Plasma Cytokine Levels After A 3 Hour Run

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Abstract

Sixteen experienced marathoners ran on treadmills for 3 h at~ 70% maximal oxygen consumption (V˙o2max) on two occasions while receiving 1 l/h carbohydrate (CHO) or placebo (Pla) beverages. Blood and vastus lateralis muscle biopsy samples were collected before and after exercise. Plasma was analyzed for IL-6, IL-10, IL-1 receptor agonist (IL-1ra), IL-8, cortisol, glucose, and insulin. Muscle was analyzed for glycogen content and relative gene expression of 13 cytokines by using real-time quantitative RT-PCR. Plasma glucose and insulin were higher, and cortisol, IL-6, IL-10, and IL-1ra, but not IL-8, were significantly lower postexercise in CHO vs. Pla. Change in muscle glycogen content did not differ between CHO and Pla ($P = 0.246$). Muscle cytokine mRNA content was detected preexercise for seven cytokines in this order (highest to lowest): IL-15, TNF-α, IL-8, IL-1β, IL-12p35, IL-6, and IFN-γ. After subjects ran for 3 h, gene expression above prerun levels was measured for five of these cytokines: IL-1β, IL-6, and IL-8 (large increases), and IL-10 and TNF-α (small increases). The increase in mRNA (fold difference from preexercise) was attenuated in CHO (15.9-fold) compared with Pla (35.2-fold) for IL-6 ($P = 0.071$) and IL-8 (CHO, 7.8-fold; Pla, 23.3-fold; $P = 0.063$). CHO compared with Pla beverage ingestion attenuates the increase in plasma IL-6, IL-10, and IL-1ra and gene expression for IL-6 and IL-8 in athletes running 3 h at 70%V˙o2max despite no differences in muscle glycogen content.

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Carbohydrate ingestion influences skeletal muscle cytokine mRNA and plasma cytokine levels after a 3-h run

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muscle glycogen; real-time quantitative reverse transcriptase-polymerase chain reaction; cortisol; glucose

THE FIRST PUBLISHED REPORT that prolonged, intensive exercise increased plasma interleukin-6 (IL-6) occurred in 1991 (17). Multiple investigations during the 1990s (13, 15, 24) revealed that plasma levels of three anti-inflammatory cytokines [IL-6, IL-10, and IL-1 receptor agonist (IL-1ra)] were most strongly elevated after strenuous exercise. Plasma levels of IL-8, a neutrophil chemotactic and activation protein, also were reported to rise after prolonged, intense exercise (13, 24). Postexercise plasma levels are slightly increased for the proinflammatory cytokines, tumor necrosis factor-α (TNF-α) and IL-1β, with negligible changes reported for the immunomodulatory cytokines, IL-2, IL-12, interferon (IFN)-γ, and IFN-α (6, 13, 24).

Investigators have sought to determine the source of these cytokines during exercise, with most of the focus on IL-6 (6). Using innovative techniques that included one- and two-legged knee-extensor exercise, femoral vein and artery catheterization, and skeletal muscle biopsy samples for IL-6 mRNA, the team from the Copenhagen Muscle Research Center has shown that IL-6 is released by muscle and peritendon in the contracting limb and by the brain, whereas the liver clears IL-6 during exercise (6, 8, 9, 18, 21–23). Blood monocytes do not appear to be the source of IL-6 during exercise (6, 20).

In a series of investigations during the mid- to late 1990s, our research team showed that plasma concentrations of IL-6, IL-10, and IL-1ra were lower in endurance athletes who ingested a 6% carbohydrate compared with placebo beverage (~1 l/h of exercise) (12, 14, 15). Carbohydrate ingestion harnessed other inflammatory indicators, including neutrophil and monocyte blood cell counts, and granulocyte/monocyte phagocytosis and oxidative burst activity (12, 14, 15). Earlier studies had shown that carbohydrate compared with placebo ingestion improved maintenance of blood glucose levels, causing a decrease in release of ACTH, cortisol, and epinephrine (11). Given the potential link between stress hormones and cytokine production during exercise, we hypothesized that carbohydrate ingestion attenuated anti-inflammatory cytokine production during exercise through a blood glucose-sympathoadrenal pathway (12, 14, 15).

Others have sought to clarify the underlying mechanisms in the relationship between IL-6 and carbohy-

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drate metabolism. Starkie et al. (19) reported that skeletal muscle IL-6 mRNA expression and the rate of decrease in muscle glycogen content were unaffected by carbohydrate ingestion in seven men who ran or cycled for 60 min. In this study, the plasma IL-6 response was blunted by carbohydrate ingestion, suggesting that either IL-6 production and subsequent release from skeletal muscle was attenuated and/or that IL-6 production from tissues other than skeletal muscle was reduced. Steensberg et al. (21) had seven men perform 5 h of two-legged knee-extensor exercise, with one leg reduced in muscle glycogen content. Muscle IL-6 mRNA expression and IL-6 release were augmented in the glycogen-depleted leg. Other studies indicated that IL-6 mRNA expression, the transcription rate of the IL-6 gene, and the release of IL-6 from the working muscle were enhanced during exercise in the glycogen-depleted state (8, 9, 21). Although epinephrine infusion does not increase plasma IL-6 to the same levels seen during exercise (23), Helge et al. (8) showed that epinephrine and several other factors, including exercise intensity, muscle glucose uptake, and muscle glycogen content, were related to IL-6 release during exercise.

It will require further research to sort out which of these parameters most consistently causes IL-6 release and the release of other cytokines in a variety of exercise conditions. The leg extensor exercise model used in many of the studies thus far involves concentric muscle activity at relatively low pulmonary maximal oxygen consumption ($\dot{V}O_{2\,max}$) and plasma IL-6 levels compared with prolonged running (6, 9, 21, 22). The highest plasma levels of IL-6, IL-10, IL-1ra, and IL-8 are attained after high-intensity running that is sustained for ≥90 min (6, 14, 24). A concentration apex is achieved at 3–4 h with little or no further increase measured even when the duration is extended to 27 h during ultramarathons (13–17). We designed a study to test the hypothesis that carbohydrate blunts plasma levels of inflammatory cytokines by influencing muscle glycogen content and cytokine gene expression during prolonged, intensive exercise. Experienced marathoners ran for 3 h on treadmills under carbohydrate and placebo conditions with muscle and blood samples collected pre- and postexercise. The muscle samples were tested for gene expression of 13 different cytokines. We hypothesized that carbohydrate ingestion would influence the rate of decrease in muscle glycogen and, as a consequence, gene expression for several inflammatory cytokines.

MATERIALS AND METHODS

Subjects. Sixteen experienced marathon runners were recruited through a letter of invitation. Male and female runners ranging in age from 25 to 60 yr were accepted into the study if they had run at least one competitive marathon, had been training for marathon races for 2 yr or more, and were willing to adhere to all aspects of the research design. Informed consent was obtained from each subject, and the experimental procedures were in accordance with the policy statements of the institutional review board of Appalachian State University.

Research design. Three to six weeks before the first test session, subjects reported to the Appalachian State University Human Performance Laboratory for orientation and measurement of body composition and cardiorespiratory fitness. Body composition was assessed by hydrostatic weighing, and $\dot{V}O_{2\,max}$ was determined by using a graded maximal protocol adapted for runners as described in earlier studies from our group (14–16). Oxygen uptake and ventilation were measured by using the MedGraphics CPX metabolic system (MedGraphics, St. Paul, MN). Heart rate was measured by using a chest heart rate monitor (Polar Electro, Woodbury, NY). Basic demographic and training data were obtained through a questionnaire.

Subjects functioned as their own controls and came back to the lab for two 3-h runs on the treadmill and ingested carbohydrate or placebo beverages in a randomized, counterbalanced design. Subjects reported to the lab at 6:00 AM not having ingested energy in any form for at least 8 h. The two runs were 4–6 wk apart, and three to four marathoners ran on treadmills in the same room at the same time on the same day of the week (7:00–10:00 AM, Saturday), with metabolic measures collected every 30 min to verify workload (using the same equipment used during baseline testing). On the test days, subjects received 6% carbohydrate or placebo beverages 15–30 min pre-run (12 ml/kg) and during the 3-h run ($4\ ml \cdot kg^{-1} \cdot 15\ min^{-1}$). Beverages were supplied by the Gatorade Sports Science Institute (Barrington, IL) as in earlier studies (12, 14, 15). The carbohydrate and placebo beverages were identical in appearance and taste. The two fluids were identical in sodium (~19.0 meq/l) and potassium (~3.0 eq/l) concentration and pH (~3.0). No other beverages or food were ingested during this time. Blood, saliva, and skeletal muscle biopsy samples were collected ~30 min prerun and immediately postrun.

Five control subjects were recruited who sat in the lab during the 3-h treadmill run sessions. Blood and muscle samples were collected from these subjects before and after sitting in the lab by using the same procedures as described for the runners. These samples were analyzed to control for the effect of laboratory measurements and, in particular, the muscle biopsy procedure and the diurnal effect on plasma cortisol.

Runners agreed to avoid the use of large-dose vitamin/mineral supplements (above 100% of recommended dietary allowances), herbs, and medications known to affect immune function for 1 wk before each run. During orientation, a dietitian instructed the runners to follow a diet high in carbohydrate during the 3 days before each 3-h run (through use of a food list) and record intake in a food record. The food records were analyzed by using a computerized dietary assessment program (Food Processor, ESHA Research, Salem, Oregon).

Skeletal muscle biopsies. Skeletal muscle biopsy samples were acquired before and after exercise after blood/saliva sample collection. The exact same procedures were utilized pre- and postexercise, with incisions made in the same thigh ~3 cm apart. During the second 3-h treadmill run, samples were collected from the opposite thigh. Local anesthesia (1% xylocaine) was injected subcutaneously into the vastus lateralis. A muscle biopsy sample was then obtained by using the percutaneous needle biopsy procedure modified to include suction (1, 5). Muscle biopsy samples were divided into two pieces and immediately frozen in liquid nitrogen. Samples were stored at −80°C until subsequent analysis.

Muscle glycogen analysis. Samples were later freeze dried, powdered, and dissected free of connective tissue, blood, and other non-muscle constituents. A portion of the muscle was extracted with acid, neutralized, and glucosyl units were analyzed enzymatically in triplicate by using a spectrophotometer (7).

Total RNA isolation and cDNA. Synthesis Procedures for RNA isolation were adapted from Carson and Booth (2). Briefly, skeletal muscle tissue was homogenized under liquid nitrogen with a Polytron, and total RNA was extracted by using the guanidine thiocynate method of Chomczynski and Sacchi (3) with Trizol reagent (Life Technologies, GIBCO-BRL). The extracted RNA (2.5 μl of sample) was dissolved in diethylpyrocarbonate-treated water and quantified spectrophotometrically at a 260-nm wavelength. RNA was reverse transcribed into cDNA in a 100-μl reaction volume containing 34.75 μl of RNA (1.5 μg) in RNase-free water, 10 μl of 10× RT buffer, 22 μl of 25 mM $MgCl_2$, 20 μl of deoxyNTP (dNTP) mixture, 5 μl of random hexamers, 2 μl of RNase inhibitor, and 6.25 μl of multiscribe RT (50 U/μl). Reverse transcription was performed at 25°C for 10 min, 37°C for 60 min, and 95°C for 5 min, followed by quick chilling on ice, and stored at −20°C until subsequent amplification.

Quantitative real-time RT-PCR analysis. Quantitative real-time RT-PCR analysis was done as per manufacturer's instructions (Applied Biosystems) by using predeveloped assay reagents (IL-6) and Taqman cytokine gene expression plate 1 (IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-8, IL-10, IL-12p35, IL-12p40, IFN-γ, IL-15, and TNF-α). DNA amplification was carried out in 25 μl of Taqman Universal PCR Master Mix (AmpliTaq Gold DNA polymerase, passive reference 1, buffer, dNTPs, AmpErase UNG), 2 μl of cDNA, 18 μl of RNase-free water, and 2.5 μl of 18S primer (VIC) and 2.5 μl of primer (FAM) (for endogenous reference and target cytokine) in a final volume of 50 μl/well. Human control RNA (calibrator RNA) was also used and served as a calibrator for each plate. Samples were loaded in a MicroAmp 96-well reaction plate. For the Taqman cytokine gene expression plate 1, all 12 cytokine primers and probes were dried and preloaded in a MicroAmp 96-well reaction plate by the manufacturer. Two dye layers were used for the quantification of the target cytokine mRNA (FAM) and the 18S ribosomal RNA endogenous control (VIC). Plates were run by using ABI sequence detection system. After 2 min at 50°C and 10 min at 95°C, plates were coamplified by 50 repeated cycles of which one cycle consisted of 15 s denaturing step at 95°C and 1 min annealing/extending step at 60°C. Data were analyzed by ABI software by using the cycle threshold (C_T), which is the value calculated and based on the time (measured by PCR cycle number) at which the reporter fluorescent emission increases beyond a threshold level (based on the background fluorescence of the system) (25), and it reflects the cycle number at which the cDNA amplification is first detected. This method was able to detect our endogenous control (18S) and genes of interest in a single well. The primers for 18S were limited to ensure that adequate amounts of reagents were available for amplification of both genes. 18S was detected in the same well as the cytokine gene of interest because the reporter dyes, which attached to the Taqman probes, fluoresce at different wavelengths. Detectable cytokine mRNA was determined by using the change in C_T (ΔC_T) values for each muscle sample. A ΔC_T value of 26 (FAM C_T minus VIC C_T) was considered the minimally detectable concentration in this assay.

Samples were initially run in duplicate on three cytokine plates and one IL-6 plate to determine intra-assay coefficient of variance (CV) as well as interassay CV. Intra-assay CVs were 1.65, 2.40, and 2.09% for the FAM, VIC, and ΔC_T, respectively. Interassay CVs were 3.95 and 5.73% for the cytokine plate and IL-6, respectively. On the basis of this initial data, the remaining samples were run in singles.

Calculations for relative quantification. Quantification of cytokine gene expression was calculated by using the ΔC_T method as described by Livak and Schmittgen (10). This method uses a single sample, the calibrator sample, for comparison of every unknown sample's gene expression. This method of analysis and quantification has been shown to give similar results as the standard curve method (25). Briefly, ΔC_T [$C_{T(FAM)} - C_{T(VIC)}$] was calculated for each sample and calibrator. $\Delta\Delta C_T$ [$\Delta C_{T(calibrator)} - \Delta C_{T(sample)}$] was then calculated for each sample, and relative quantification was calculated as $2\Delta\Delta C_T$. Initial exclusion criteria consisted of FAM $C_T \geq 40$ and VIC $C_T \geq 23$. All samples had FAM C_T values of between 25 and 38. A one-unit change in C_T reflects a twofold change in mRNA content. Lower C_T values reflect higher mRNA levels. Detectable cytokine gene expression (mRNA) was defined as a ΔC_T value of ≤26. This reflects a FAM C_T value of ≤36 with an average VIC C_T value that was very consistent at ~10.2. The unusually high levels of IL-10 and TNF-α mRNA in the human calibrator mRNA resulted in a very low $\Delta\Delta C_T$ (and resulting relative fold difference from calibrator) value for these cytokines in the muscle samples, which does not necessarily indicate a low level of IL-10 and TNF-α mRNA in the samples.

Blood cell counts, hormones, and glucose. Blood samples were drawn from an antecubital vein with subjects in the seated position. Routine complete blood counts were performed by our clinical hematology laboratory and provided leukocyte subset counts, hemoglobin, and hematocrit. Other blood samples were centrifuged in sodium heparin tubes, and plasma was aliquoted and then stored at −80°C. Plasma cortisol was assayed in duplicate by using the competitive solid-phase ^{125}I radioimmunoassay technique (Diagnostic Products, Los Angeles, CA). Radioimmunoassay kits were also used to determine plasma concentrations of insulin in duplicate according to manufacturer's instructions (Diagnostic Products). Plasma was analyzed spectrophotometrically for glucose. Plasma volume changes were estimated by using the method of Dill and Costill (4).

Plasma cytokine measurements. Total plasma concentrations of IL-1ra, IL-6, IL-8, and IL-10 were determined by using quantitative sandwich ELISA kits provided by R&D Systems (Minneapolis, MN). All samples and provided standards were analyzed in duplicate. A high-sensitivity kit was used to analyze IL-6 in the prerace plasma samples. A standard curve was constructed by using standards provided in the kits, and the cytokine concentrations were determined from the standard curves by using linear regression analysis. The assays were a two-step "sandwich" enzyme immunoassay in which samples and standards were incubated in a 96-well microtiter plate coated with polyclonal antibodies for the test cytokine as the capture antibody. After the appropriate incubation time, the wells were washed, and a second detection antibody conjugated to either alkaline phosphatase (IL-6 high sensitivity) or horseradish peroxidase (IL-1ra, IL-6, IL-8, IL-10) was added. The plates were incubated and washed, and the amount of bound enzyme-labeled detection antibody was measured by adding a chromogenic substrate. The plates were then read at the appropriate wavelength (450 − 570 nm for IL-1ra, IL-6, IL-8, and IL-10; 490 − 650 nm for IL-6 high sensitivity). The minimum detectable concentration of IL-1ra was <22 pg/ml, IL-6 <0.70 pg/ml, IL-6 high sensitivity <0.094 pg/ml, IL-8 <10 pg/ml, and IL-10 <3.9 pg/ml. Because of the lack of high-sensitivity kits for

Table 1. *Subject characteristics*

Age, yr	50.0 ± 1.5
Stature, m	1.76 ± 0.02
Body mass, kg	73.2 ± 3.2
Body composition, %fat	16.6 ± 1.0
Heart rate (maximal), beats/min	168 ± 3
$\dot{V}O_{2max}$, ml·kg^{-1}min^{-1}	45.0 ± 1.7
Respiratory exchange ratio (maximal)	1.11 ± 0.02
Minute ventilation, maximal, l/min	118 ± 6
Training distance, km/wk	62.6 ± 7.6

Values are means ± SE of n = 16 subjects.

IL-8 and IL-10, we extrapolated data below the minimum detectable level by using a software program suited to this task. Pre- and postexercise samples for IL-8, IL-10, and IL-1ra were analyzed on the same assay plate to decrease inter-kit assay variability.

Salivary samples. Unstimulated saliva was collected by expectoration into 15-ml plastic, sterilized vials for 4 min. Participants were urged to pass as much saliva as possible into the vials during the 4-min timed session. The saliva samples were frozen at −80°C until analysis. Saliva volume was measured to the nearest 0.1 ml, and saliva total protein was quantified by using the Coomassie protein assay reagent. Salivary IgA was measured by enzyme-linked immunosorbent assay according to the procedures adapted from the Hunter Immunology Unit (Royal Newcastle Hospital, Newcastle, Australia). Data were expressed as concentration of sIgA relative to total protein concentration (μg/mg).

Statistical analysis. Statistical significance was set at the $P \leq 0.05$ level, and values are expressed as means ± SE. Performance measures were compared under carbohydrate and placebo conditions by using paired t-tests. Data in Tables 3–5 and all figures were analyzed by using a 2 (carbohydrate and placebo conditions) × 2 (times of measurement) repeated-measures ANOVA. If the condition × time interaction P value was ≤0.05, the change from pre- to postexercise values was calculated and compared between conditions by using paired t-tests. Pearson product-moment correlations were used to test the relationship between changes in plasma and muscle measures.

RESULTS

Table 1 summarizes subject characteristics for the 16 runners (12 men, 4 women) completing all phases of the study. Data for the male and female runners were combined because no significant differences were measured for the hormonal and immune data reported in this paper. The marathon runners in this study were highly experienced and committed to regular training and racing but were still well below elite status. The

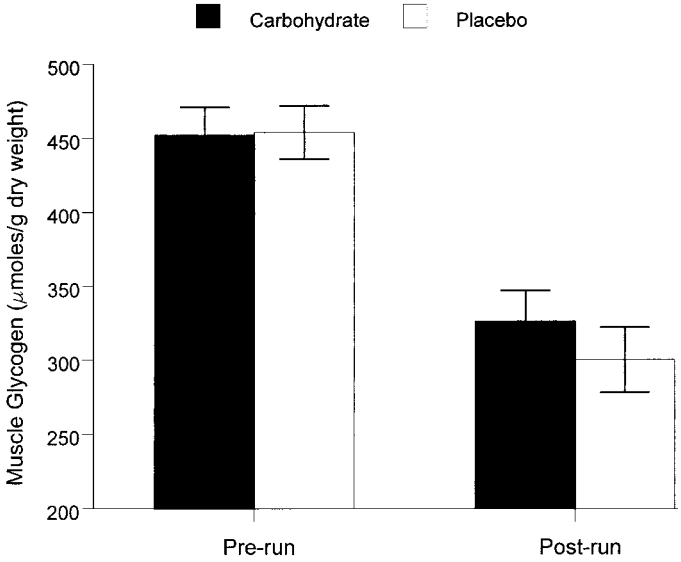

Fig. 1. The decrease in skeletal muscle glycogen content did not differ in 16 runners under carbohydrate and placebo conditions after a 3-h treadmill run at ~70% maximal oxygen consumption ($\dot{V}O_{2max}$) [$F(1,15) = 1.46$, $P = 0.246$]

treadmill test data indicate a high degree of cardiorespiratory fitness for this age group. Carbohydrate intake during the 3 days before the 3-h treadmill runs did not differ significantly between carbohydrate and placebo conditions (58.8 ± 3.1 and 61.8 ± 2.1% of total energy intake, respectively; $P = 0.271$).

Oxygen consumption was measured six times during the 3-h treadmill runs and averaged 30.9 ± 1.0 (69.1 ± 1.2% $\dot{V}O_{2\,max}$) and 31.4 ± 0.9 ml · kg^{-1} · min^{-1} (70.3 ± 1.3% $\dot{V}O_{2\,max}$) in the carbohydrate and placebo conditions, respectively ($P = 0.176$). Heart rates during the 3-h treadmill runs averaged 81.4 ± 1.1 and 81.5 ± 1.3% maximal heart rate in the carbohydrate and placebo conditions, respectively ($P = 0.922$). Plasma volume shift was negligible and did not differ between carbohydrate and placebo conditions (1.9 ± 0.3 and 1.5 ± 0.6%, respectively; $P = 0.597$).

The increase in blood cell counts for total leukocytes, granulocytes, and monocytes was attenuated in the carbohydrate compared with placebo condition (Table 2). Mean granulocytosis was 94 and 182%, and monocytosis was 2 and 43% in the carbohydrate and placebo conditions, respectively ($P < 0.001$). Postexercise levels of blood lymphocytes were significantly lower in the

Table 2. *Change in blood leukocyte subset counts in 16 runners under carbohydrate and placebo conditions after a 3-h treadmill run at ~70% $\dot{V}O_{2max}$*

	Carbohydrate		Placebo		
Variable ($10^9 \cdot l^{-1}$)	Prerun	Postrun	Prerun	Postrun	P Values: Interaction
Total leukocytes	5.21 ± 0.29	7.62 ± 0.55*	4.99 ± 0.29	10.02 ± 0.75	<0.001
Granulocytes	2.99 ± 0.22	5.80 ± 0.47*	2.78 ± 0.24	7.83 ± 0.63	<0.001
Monocytes	0.44 ± 0.03	0.45 ± 0.04*	0.42 ± 0.03	0.60 ± 0.06	<0.001
Lymphocytes	1.57 ± 0.08	1.28 ± 0.12*	1.61 ± 0.10	1.50 ± 0.12	0.036

Values are means ± SE. *Significant difference in change in carbohydrate compared with placebo condition ($P < 0.05$).

Table 3. *Change in plasma glucose, insulin, cytokines, and salivary IgA to protein ratio in 16 runners under carbohydrate and placebo conditions after a 3-h treadmill run at ~70% $\dot{V}O_{2max}$*

Variable	Carbohydrate		Placebo		*P* Values: Interaction
	Prerun	Postrun	Prerun	Postrun	
Glucose, mmol/l	3.84 ± 0.2	5.17 ± 0.2*	3.75 ± 0.1	4.02 ± 0.2	0.003
Insulin, pmol/l	63.6 ± 6.7	126 ± 26*	77.0 ± 10.6	67.2 ± 8.8	0.008
IL-10, pg/ml	4.09 ± 1.08	13.3 ± 3.7*	3.79 ± 1.02	20.4 ± 5.7	0.013
IL-1ra, pg/ml	227 ± 25	306 ± 32*	197 ± 24	418 ± 58	0.002
IL-8, pg/ml	6.51 ± 1.09	12.2 ± 1.1	7.91 ± 1.02	13.3 ± 1.6	0.873
sIgA:protein, μg/mg	568 ± 171	309 ± 40	627 ± 135	302 ± 47	0.624

Values are means ± SE. *Significant difference in change in carbohydrate compared with placebo condition ($P < 0.05$).

carbohydrate compared with placebo condition ($P = 0.036$).

The skeletal muscle biopsy procedure was initiated immediately postrun, with samples removed from the vastus lateralis 14.1 ± 0.8 and 13.1 ± 1.0 min postrun in the carbohydrate and placebo conditions, respectively ($P = 0.419$). Muscle glycogen decreased significantly under both carbohydrate (28%) and placebo (34%) conditions at a similar rate [$F(1,15) = 1.46$, $P = 0.246$] (Fig. 1). The pattern of change in plasma glucose and insulin was significantly different between conditions, with postrun levels higher in the carbohydrate condition (Table 3). Figure 2 indicates that plasma cortisol decreased 60% in the five sitting controls, compared with 24 and 3.4% in the runners during carbohydrate and placebo conditions, respectively [$F(1,15) = 6.26$, $P = 0.024$]. The pattern of increase in plasma IL-6, IL-10, and IL-1-ra, but not IL-8, was significantly different between conditions, with postrun levels lower in the carbohydrate condition (Fig. 3 and Table 3). Salivary IgA-to-protein ratio decreased 46 and 52% in the carbohydrate and placebo conditions, respectively (interaction effect, $P = 0.624$) (Table 3).

In these subjects, muscle cytokine mRNA content was detected preexercise for seven cytokines in this order (highest to lowest mRNA concentration): IL-15, TNF-α, IL-8, IL-1β, IL-12p35, IL-6, and IFN-γ (Table 4). IL-10 mRNA content in preexercise muscle samples was just below the minimally detectable concentration ($\Delta C_T = 26.4$). IL-1β, IL-6, IL-8, IL-10, and TNF-α were significantly increased with exercise ($P < 0.05$) (Table 5 and Figs. 4–6). The increase in mRNA (difference from preexercise) was attenuated in carbohydrate (15.9-fold) compared with placebo (35.2-fold) for IL-6 ($P = 0.071$) and IL-8 (carbohydrate, 7.8-fold; placebo, 23.3-fold; $P = 0.063$), but not IL-1β (Figs. 4–6). As depicted in Figs. 4–6, the muscle biopsy procedure did not increase gene expression for IL-6, IL-8, and IL-1β in the sitting controls. No significant increase in gene expression was measured in the sitting controls for other cytokines listed in Table 4 except for TNF-α (0.031 ± 0.003, 0.046 ± 0.008-fold difference from calibrator, $P = 0.041$, first and second samples, respectively). Plasma levels for IL-6, IL-10, IL-8, and IL-1ra did not increase significantly in the sitting controls (data not shown).

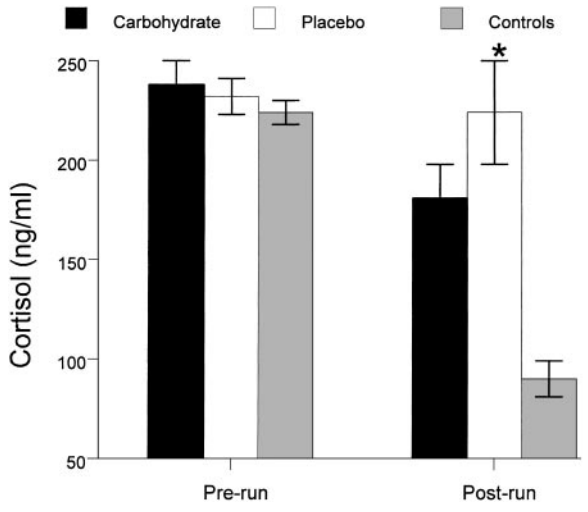

Fig. 2. Plasma cortisol decreased 60% in the 5 sitting controls compared with 24 and 3.4% in the runners during carbohydrate and placebo conditions, respectively [$F(1,15) = 6.26$, $P = 0.024$]. *Significant difference in change in carbohydrate compared with placebo condition ($P < 0.05$).

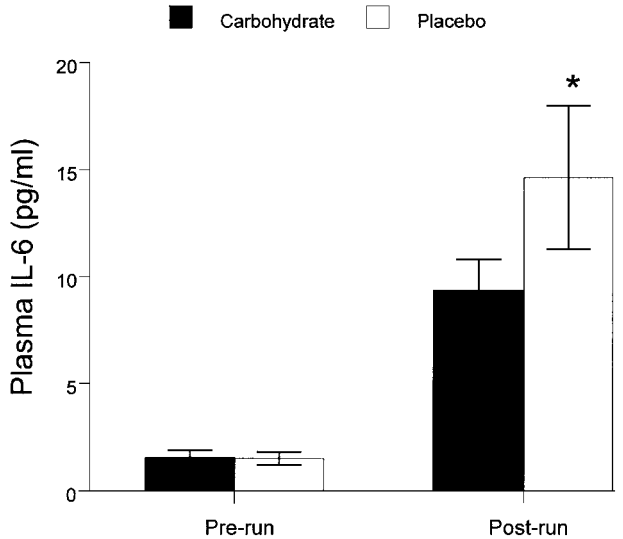

Fig. 3. Pattern of increase in plasma IL-6 was significantly different between conditions, with postrun levels being lower in the carbohydrate condition. *Significant difference in change in carbohydrate compared with placebo condition ($P < 0.05$).

Table 4. *Detectable cytokine mRNA in preexercise skeletal muscle samples*

Cytokine	ΔC_T
IL-15	18.0 ± 0.5
TNF-α	22.3 ± 0.5
IL-8	23.6 ± 0.5
IL-1β	23.7 ± 0.5
IL-12p35	24.4 ± 0.5
IL-6	25.4 ± 0.3
IFN-γ	25.7 ± 0.5

Values are means ± SE of $n = 31$ subjects. Detectable cytokine mRNA was determined by using the change in cycle threshold (ΔC_T) values for each cytokine mRNA in each muscle sample. A ΔC_T value of 26 (FAM C_T minus VIC C_T) was considered the minimally detectable concentration in this assay. The VIC C_T values were very consistent at ~10.2. For comparison purposes for these cytokines, the first 1-unit increase in ΔC_T reflects a twofold decrease in mRNA content, and then fourfold, eightfold, etc. For example, it is reasonable to suggest that the TNF-α mRNA content is approximately eightfold greater than the IL-6 mRNA content in the preexercise muscle samples.

Change in skeletal muscle IL-6, IL-8, and IL-1β mRNA content was correlated with each other and changes in all other variables measured in this study for carbohydrate and placebo conditions. In both the carbohydrate and placebo conditions, IL-1β gene expression correlated significantly with IL-6 gene expression ($r = 0.79$, $P < 0.001$, and $r = 0.91$, $P < 0.001$, respectively). In the placebo but not the carbohydrate condition, IL-6 and IL-1β gene expression correlated significantly with IL-8 gene expression ($r = 0.90$, $P < 0.001$, and $r = 0.87$, $P < 0.001$, respectively) and change in plasma IL-8 ($r = 0.73$, $P = 0.001$, and $r = 0.86$, $P < 0.001$, respectively). In the placebo condition only, change in the blood lymphocyte count correlated significantly with IL-6, IL-1β, and IL-8 gene expression ($r = 0.65$, $P = 0.006$; $r = 0.64$, $P = 0.008$; and $r = 0.67$, $P = 0.005$, respectively). Change in muscle glycogen content, plasma glucose levels, or plasma cortisol levels did not significantly correlate with IL-6, IL-1β, or IL-8 gene expression.

DISCUSSION

Our data indicate that muscle cytokine mRNA content can be detected in samples taken from marathon runners in the resting state for IL-15, TNF-α, IL-8, IL-1β, IL-12p35, IL-6, and IFN-γ but not IL-1α, IL-2, IL-4, IL-5, IL-10, and IL-12p40. After subjects ran for 3 h, gene expression above prerun levels was measured for five of these cytokines: IL-1β, IL-6, and IL-8 (largest increases), and IL-10 and TNF-α (smaller increases). Preexercise, the concentration of mRNA for IL-10 and IL-6 was minimally detectable, and postexercise the increase in IL-10 was small compared with the large increase in IL-6.

As in previous studies from our laboratory, carbohydrate compared with placebo ingestion attenuated post-run increases in three anti-inflammatory cytokines in the plasma: IL-6, IL-1ra, and IL-10 (12, 14, 15). The increase in skeletal muscle mRNA was considerably diminished in the carbohydrate compared with placebo condition for IL-6 and IL-8, but not IL-β, despite no differences in postrun muscle glycogen levels. Carbohydrate ingestion did not alter the exercise-induced increase in plasma IL-8 but did diminish IL-8 mRNA, suggesting an additional source other than muscle for IL-8 or an alteration of production/disappearance. Carbohydrate ingestion decreased the exercise-induced increase in plasma IL-10 but did not alter the small but significant gene expression for this cyto-

Table 5. *Change in skeletal muscle cytokine mRNA content in 16 runners under carbohydrate and placebo conditions after a 3-h treadmill run at ~70% $\dot{V}O_{2max}$*

Variable	Carbohydrate		Placebo		*P* Values: Interaction; Time
	Prerun	Postrun	Prerun	Postrun	
IL-1α	0.82 ± 0.09	1.42 ± 0.38	1.06 ± 0.19	3.11 ± 1.48	0.373, 0.097
IL-2	2.10 ± 0.68	2.59 ± 0.59	1.37 ± 0.23	1.45 ± 0.31	0.581, 0.434
IL-4	0.12 ± 0.05	0.12 ± 0.05	0.16 ± 0.05	0.13 ± 0.04	0.182, 0.471
IL-5	0.39 ± 0.11	0.52 ± 0.12	0.58 ± 0.17	0.79 ± 0.18	0.717, 0.304
IL-10	0.001 ± 0.0001	0.003 ± 0.001	0.002 ± 0.0001	0.004 ± 0.001	0.267, 0.001
IL-12p35	5.45 ± 1.22	5.61 ± 1.26	6.10 ± 1.34	7.76 ± 1.97	0.444, 0.396
IL-12p40	0.27 ± 0.14	0.33 ± 0.22	0.08 ± 0.02	0.15 ± 0.03	0.946, 0.229
IL-15	112 ± 11.8	99.2 ± 11.5	145 ± 22.3	123 ± 14.2	0.630, 0.067
IFN-γ	1.54 ± 0.62	2.08 ± 1.79	2.40 ± 1.12	0.57 ± 0.17	0.126, 0.499
TNF-α	0.024 ± 0.003	0.046 ± 0.008	0.028 ± 0.005	0.057 ± 0.008	0.192, 0.001

Values (fold difference from calibrator) are means ± SE. The very low values for IL-10 and TNF-α reflect the large quantity of mRNA for these cytokines in the calibrator mRNA sample. It does not necessarily reflect a low concentration of mRNA in the experimental samples.

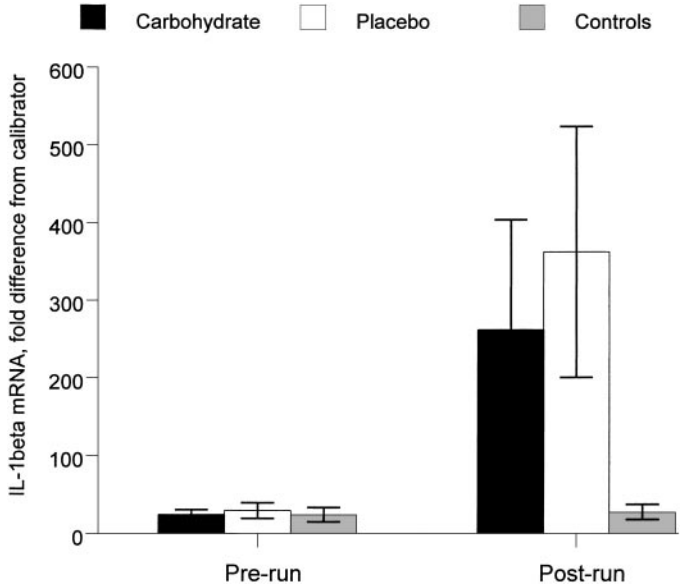

Fig. 4. Pattern of increase in muscle IL-1β mRNA expression after 3 h of running did not differ between conditions [$F(1,15) = 0.22$, $P = 0.645$]. No significant change in muscle IL-1β mRNA expression was measured in sitting controls experiencing the same muscle biopsy procedure ($P = 0.582$).

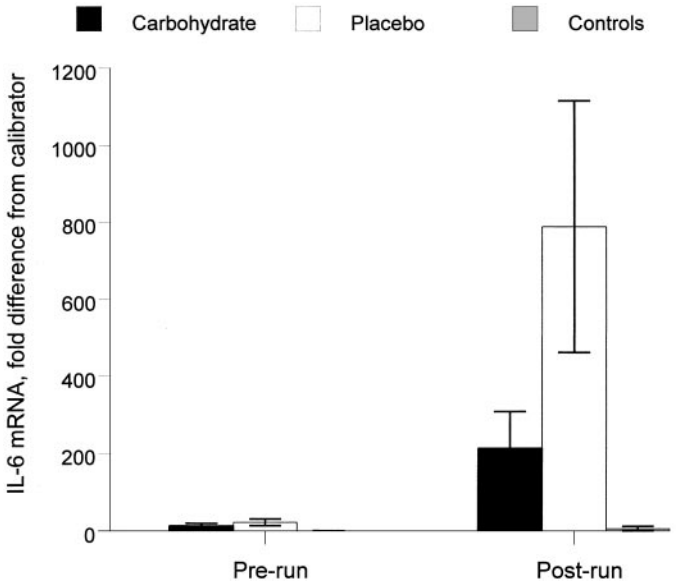

Fig. 5. Pattern of increase in muscle IL-6 mRNA expression after 3 h of running tended to differ between conditions [$F(1,15) = 3.79$, $P = 0.071$], with lower postrun expression in the carbohydrate condition. No significant change in muscle IL-6 mRNA expression was measured in sitting controls experiencing the same muscle biopsy procedure ($P = 0.381$).

kine. Both carbohydrate and exercise may exert an influence on IL-10 production in another body compartment. Exercise strongly induced IL-1β mRNA expression irrespective of carbohydrate ingestion, suggesting that this proinflammatory cytokine is countered by a rapid anti-inflammatory response that successfully combats its elevation in the plasma. In both the carbohydrate and placebo conditions, IL-1β gene expression correlated strongly with IL-6 gene expression; in the placebo condition only, IL-6 and IL-1β gene expression correlated significantly with IL-8 gene expression. These correlational data suggest that long-duration running with or without carbohydrate induces IL-1β mRNA expression but that concomitant or subsequent IL-6 and IL-8 mRNA expression is decreased when carbohydrate is ingested.

These data contrast with those of Starkie et al. (19). Skeletal muscle IL-6 mRNA expression was measured in samples obtained from seven men preexercise and increased ~20-fold after 60 min of moderate running or cycling despite no differences in postexercise muscle glycogen levels. IL-6 mRNA expression was similar whether these subjects ingested a carbohydrate beverage (12 ml/kg, 6.4% carbohydrate, ~64 g total) or water. In contrast, IL-6 mRNA expression increased ~16-fold above rest in our subjects, who ingested ~60 g carbohydrate/h compared with ~35-fold in the placebo condition during 3-h of treadmill running. Starkie et al. (19) also reported significantly diminished postexercise levels of plasma IL-6 in the carbohydrate compared with water condition and reasoned that either IL-6 production and subsequent release from skeletal muscle was attenuated, and/or that IL-6 production from tissues other than skeletal muscle was reduced. In our study, post-run plasma IL-6 was about twice as high as reported by Starkie et al. (19) and increased 507% above prerun levels in the carbohydrate condition compared with a 875% increase in the placebo condition. Taken together, our data indicate that the diminished postrun plasma IL-6 concentration is at least in part due to an attenuation of skeletal muscle IL-6 mRNA expression. Our data may differ

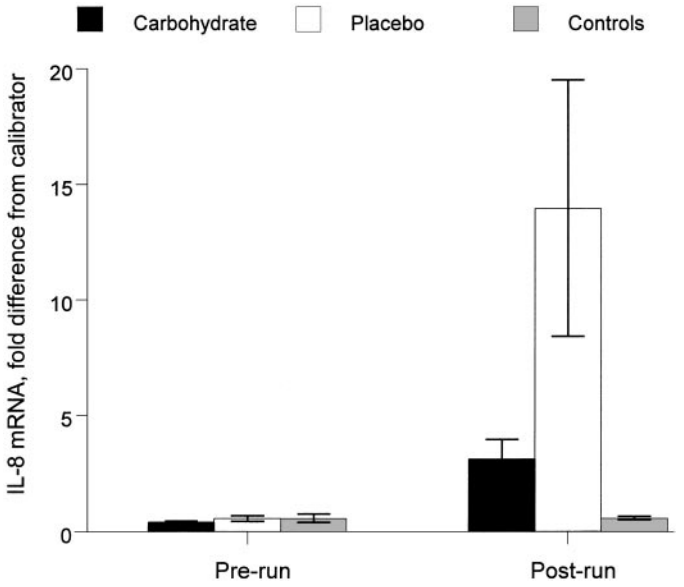

Fig. 6. Pattern of increase in muscle IL-8 mRNA expression after 3 h of running tended to differ between conditions [$F(1,15) = 4.04$, $P = 0.063$], with lower postrun expression in the carbohydrate condition. No significant change in muscle IL-8 mRNA expression was measured in sitting controls experiencing the same muscle biopsy procedure ($P = 0.994$).

from those of Starkie et al. (19) because of the contrast in metabolic workload and duration of exercise (3 h compared with 1 h).

Febbraio and Pedersen (6) have hypothesized that muscle glycogen availability may influence key signaling molecules (presently undetermined) to enhance IL-6 gene transcription within skeletal muscle during altered homeostasis. Keller et al. (9) showed that 180 min of two-legged knee-extensor exercise activated transcription of the skeletal muscle IL-6 gene and that this response was enhanced under conditions in which muscle glycogen concentrations were low. Our data indicate that IL-6 mRNA expression is influenced by additional factors that can be altered by carbohydrate ingestion. Helge et al. (8), using a knee-extension exercise model and catheters in the femoral artery and veins, showed that thigh IL-6 release was significantly related to exercise intensity, thigh glucose uptake, arterial plasma epinephrine concentration, and postexercise glycogen concentration. Multiple studies have shown that epinephrine is an inducer of cytokine release during exercise (for review, see Ref. 24) and that carbohydrate ingestion blunts epinephrine release by increasing blood glucose (11). We did not measure epinephrine in this study but did show a consistent relationship between the blood lymphocyte count and IL-6, IL-1β, and IL-8 gene expression in the placebo condition. Epinephrine plays a key role in exercise-induced changes in lymphocyte number (22). Thus it is likely that, despite no differences in postrun muscle glycogen levels, IL-6 mRNA and IL-8 mRNA expression in our subjects was diminished in the carbohydrate compared with placebo condition due in part to differences in blood glucose and epinephrine levels.

We conclude that carbohydrate compared with placebo ingestion during a 3-h treadmill run attenuates plasma levels of IL-1ra, IL-6, and IL-10 and muscle gene expression for IL-6 and IL-8. Blood and muscle samples were obtained as quickly as possible postexercise, and additional samples during recovery should provide important information in future studies. The 3-h treadmill run in both the carbohydrate and placebo conditions induced gene expression within the muscle for two primary proinflammatory cytokines IL-1β and TNF-α. IL-6 and IL-8, which are often considered to be components of the secondary inflammatory cascade, were also expressed, but to a smaller degree within the carbohydrate condition. Anti-inflammatory indicators, including plasma IL-1ra, IL-10, and cortisol, were also decreased within the carbohydrate condition. Together, these data suggest that carbohydrate ingestion attenuates the secondary but not the primary proinflammatory cascade, decreasing the need for immune responses related to anti-inflammation.

We appreciate the assistance of David Brock, Jay Cranston, Sudhir Dhobale, Blake Justice, Greg Harris, David O'Connor, Steve Rossi, and Shawn Swick with this study.

This study was supported by a grant from the Gatorade Sports Science Institute.

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