Postexercise Carbohydrate–Protein Supplementation Improves Subsequent Exercise Performance and Intracellular Signaling for Protein Synthesis

Lisa Ferguson-Stegall, Erin L. McCleave, Zhenping Ding, Phillip G. Doerner III, Bei Wang, Yi-Hung Liao, Lynne Kammer, Yang Liu, Jungyun Hwang, Benjamin M. Dessard, and John L. Ivy

Exercise Physiology and Metabolism Laboratory, Department of Kinesiology, University of Texas at Austin, Austin, Texas

Abstract

Ferguson-Stegall, L, McCleave, EL, Ding, Z, Doerner III, PG, Wang, B, Liao, Y-H, Kammer, L, Liu, Y, Hwang, J, Dessard, BM, and Ivy, JL. Postexercise carbohydrate–protein supplementation improves subsequent exercise performance and intracellular signaling for protein synthesis. *J Strength Cond Res* 25(5): 1210–1224, 2011–Postexercise carbohydrate–protein (CHO + PRO) supplementation has been proposed to improve recovery and subsequent endurance performance compared to CHO supplementation. This study compared the effects of a CHO + PRO supplement in the form of chocolate milk (CM), isocaloric CHO, and placebo (PLA) on recovery and subsequent exercise performance. Ten cyclists performed 3 trials, cycling 1.5 hours at 70% $\dot{V}O_2$max plus 10 minutes of intervals. They ingested supplements immediately postexercise and 2 hours into a 4-hour recovery. Biopsies were performed at recovery minutes 0, 45, and 240 (R0, R45, REnd). Postrecovery, subjects performed a 40-km time trial (TT). The TT time was faster in CM than in CHO and in PLA (79.43 ± 2.11 vs. 85.74 ± 3.44 and 86.92 ± 3.28 minutes, $p \leq 0.05$). Muscle glycogen resynthesis was higher in CM and in CHO than in PLA (23.58 and 30.58 vs. 7.05 $\mu mol \cdot g^{-1}$ wet weight, $p \leq 0.05$). The mammalian target of rapamycin phosphorylation was greater at R45 in CM than in CHO or in PLA (174.4 ± 36.3 vs. 131.3 ± 28.1 and 73.7 ± 7.8% standard, $p \leq 0.05$) and at REnd in CM than in PLA (94.5 ± 9.9 vs. 69.1 ± 3.8%, $p \leq 0.05$). rpS6 phosphorylation was greater in CM than in PLA at R45 (41.0 ± 8.3 vs. 15.3 ± 2.9%, $p \leq 0.05$) and REnd (16.8 ± 2.8 vs. 8.4 ± 1.9%, $p \leq 0.05$). FOXO3A phosphorylation was greater at R45 in CM and in CHO than in PLA (84.7 ± 6.7 and 85.4 ± 4.7 vs. 69.2 ± 5.5%, $p \leq 0.05$). These results indicate that postexercise CM supplementation can improve subsequent exercise performance and provide a greater intracellular signaling stimulus for PRO synthesis compared to CHO and placebo.

Key Words muscle glycogen, cycling, mTOR, rpS6, FOXO3A, endurance performance

Introduction

Recovering from strenuous endurance exercise is central to the ability to perform at one's best day after day in training sessions and competitive events. Endurance athletes must often perform multiple bouts of prolonged, strenuous activity with little time to recover in between. Studies of subsequent endurance exercise performance performed after recovering from a previous bout have reported significantly increased endurance time to exhaustion (TTE) in the subsequent exercise bout with carbohydrate–protein (CHO + PRO) supplementation compared to CHO alone (3,35,46,57). The improvements in performance have been associated with a greater recovery of muscle glycogen during the recovery period between the 2 bouts in CHO + PRO compared to in a nonisocaloric CHO beverage (4,57) and with reduced muscle damage (46). However, not all investigations have demonstrated a significant difference in subsequent TTE (37,43–45) or time trial (TT) performance (4,7,8) between CHO + PRO and CHO treatments.

In addition to the importance of postexercise supplementation in restoring muscle glycogen and attenuating muscle damage for improved recovery, nutrients play a key role in facilitating muscle PRO synthesis. Strenuous exercise stimulates PRO synthesis and PRO breakdown, and the balance between degradation and synthesis is largely mediated by nutrient availability, especially that of amino acids (AAs), and activation of the mammalian target of rapamycin (mTOR) signal transduction pathway. mTOR is a serine-threonine kinase that integrates signals from nutrients, skeletal muscle

Address correspondence to John L. Ivy, johnivy@mail.utexas.edu.

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contraction, and growth factors, functioning as a crucial regulator of PRO synthesis (31,53). Because the mTOR pathway is critical for PRO synthesis and cell growth, it is important to optimize activation of this pathway to maximize training adaptations. Although postexercise CHO + PRO supplementation has been shown to alter the phosphorylation of signaling PROs related to PRO synthesis (20,33), the effects when compared to CHO only and placebo (PLA) in an endurance exercise model have not been well characterized, nor has muscle PRO degradation-specific signaling been characterized in response to CHO + PRO supplementation postendurance exercise.

Although there are many CHO + PRO supplements commercially available, several recent investigations have used low-fat chocolate milk (CM) as a postendurance exercise CHO + PRO supplement. Pritchett et al. compared CM and a commercially available CHO + PRO postexercise supplement and found no difference between the 2 in markers of muscle damage and in TTE in a subsequent bout of exercise (40). This finding was in agreement with that of another investigation that compared CM to a CHO-replacement beverage and a fluid-replacement beverage and found that TTE after a 4-hour recovery was significantly longer in CM than in the other 2 treatments (50). Taken together, these findings suggest that CM may be a viable CHO + PRO supplement for use postexercise to aid recovery, improve subsequent exercise performance, activate signaling pathways for initiation of PRO translation, while also reducing PRO degradation signaling beyond that afforded by CHO supplementation alone. However, to our knowledge, no single investigation has examined all of these aspects together, along with markers of muscle damage, inflammation, and muscle glycogen resynthesis.

Therefore, the purpose of this study was to investigate the effects of a dairy-based CHO–PRO recovery supplement (CM) on postexercise muscle glycogen synthesis, activation of key signaling PROs involved in PRO synthesis (mTOR, rpS6, and eIF2Bε) and degradation (FOXO3A and ubiquitination), biochemical measures of muscle damage and inflammation and subsequent aerobic endurance performance. We hypothesized that, compared to an isocaloric CHO supplement, the CM supplement would improve recovery by increasing muscle glycogen synthesis and attenuate indicators of muscle damage and inflammation after a strenuous exercise bout and thus, improved subsequent TT performance. We also hypothesized that the CM supplement would more effectively modulate the phosphorylation of intracellular signaling PROs that lead to increased PRO synthesis and reduced PRO degradation during recovery compared to a CHO supplement or PLA.

Methods

Experimental Approach to the Problem

This study followed a randomized, double-blinded, PLA-controlled, crossover design. Subjects reported to the laboratory before the start of their experimental trials for maximal oxygen consumption ($\dot{V}O_2$max) and maximal workload (Wmax) determination. This test was performed on a VeloTron DynaFit Pro cycle ergometer (RacerMate, Seattle, WA, USA). The protocol for establishing $\dot{V}O_2$max consisted of a 4-minute warm-up, then 2-minute stages beginning at 200 W for men or 130 W for women. The workload was increased by 50 W (men) or 35 W (women) every 2 minutes until it was 350 and 200 W, respectively. After this point, the workload increased 25 W (men) or 10 W (women) every minute until the subject could not continue to pedal despite constant verbal encouragement. The criteria used to establish $\dot{V}O_2$max were a plateau in $\dot{V}O_2$ with increasing exercise intensity and respiratory exchange ratio (RER) > 1.10. The $\dot{V}O_2$max was measured using a TrueOne2400 system (ParvoMedics, Sandy, UT, USA). The subjects breathed through a Hans Rudolph (Han Rudolph, Inc., Shawnee, KS, USA) valve, with expired gases directed to a mixing chamber for analysis of oxygen (O_2) and carbon dioxide (CO_2). Outputs were directed to a computer for calculation of ventilation, O_2 consumption ($\dot{V}O_2$), CO_2 production ($\dot{V}CO_2$), and RER every 15 seconds.

Maximum power output in Watts was calculated from the $\dot{V}O_2$max test data using the formula, adapted from Åstrand and Rodahl (1):

$$W_{\max} = (\dot{V}O_2 \max \text{ml} - 300 \text{ ml } O_2)/12.5 \text{ W} \cdot \text{ml}^{-1} \text{ } O_2.$$

The workload for each intensity level (45, 70, and 90 of $\dot{V}O_2$max) was then set as percentages of the $W_{\max}$ as follows:

$$W = ([\dot{V}O_2 \max \text{ml} \times \%\dot{V}O_2 \max \text{desired}] - 300 \text{ ml } O_2)/12.5 \text{ W} \cdot \text{ml}^{-1} \text{ } O_2.$$

Three to 5 days after the $\dot{V}O_2$max test, the subjects again reported to the laboratory after an overnight 12-hour fast for a familiarization session. Using the same VeloTron cycle ergometer on which the $\dot{V}O_2$max test was performed, subjects performed a 40-minute portion of the glycogen depletion ride, including the intervals at the end, and gas collections taken during this ride allowed verification and subsequent adjustment of the calculated workloads for the experimental trials. Subjects then rested in the laboratory for 1 hour and then performed the 40-km cycling TT. No muscle or blood samples were collected during the familiarization session.

The protocol for the experimental trials is shown in Figure 1. For each trial, subjects reported to the laboratory having fasted overnight for 12 hours. After being weighed and fitted with a heart rate monitor (Cardiosport, Waterlooville, Hampshire, United Kingdom), a catheter with a 3-way stopcock and catheter extension was inserted into a forearm vein and taped in place. A resting blood sample was drawn, and resting heart rate recorded. Then, the subject mounted the cycle ergometer and began the glycogen depletion ride.

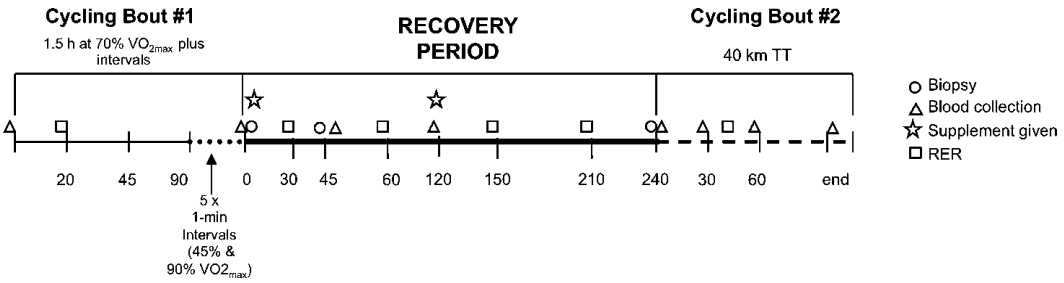

Figure 1. Experimental protocol.

Subjects cycled at 70% $\dot{V}O_2$max for 1.5 hours, followed by 1-minute alternating intervals at 45 and 90% $\dot{V}O_2$max for a total of 10 minutes. This bout was designed to deplete muscle glycogen stores. Subjects received 250 mL of water at 15-minute intervals during the ride, and floor fans were used to circulate air over the individual to minimize thermal stress. Subjects then recovered in the laboratory for 4 hours and received 1 of 3 experimental treatments immediately and at 2 hours postexercise. Muscle biopsies were performed immediately after the first exercise bout, at 45 minutes into recovery, and 4 hours after the start of recovery to assess muscle glycogen resynthesis. Blood collections were taken at the same time points as the biopsies, and at 2 hours into the recovery period. Subjects were allowed to work, read, or study at a desk set up for their use in the laboratory and were under constant observation by investigators during this time. They were provided water ad libitum during this time. At 4 time points during recovery, RER measurements were made while the subject rested quietly in a cushioned chair for 10 minutes. The catheters were flushed every 10–15 minutes throughout the recovery period.

After the recovery period, subjects completed a 40-km TT using the same VeloTron on which they performed the earlier exercise bout. The simulated TT course was designed with VeloTron 3D software (RacerMate). The subjects were instructed to cycle this fixed distance as fast as possible, and the measure of performance was the time to complete the TT. The course included rolling hills and a 3-km uphill finish. Subjects were cooled using a fan and were provided 250 mL of water at 15-minute intervals during the TT. Verbal encouragement was given consistently to all subjects, and the same investigators provided encouragement to the same subjects for each TT. Each experimental trial was separated by a minimum of 7 days but did not exceed 14 days.

The outcome measures of interest were muscle glycogen resynthesis during recovery; indicators of muscle damage and inflammation (myoglobin, creatine phosphokinase [CPK], and cytokines interleukin-6 [IL-6], IL-8, IL-10, IL-1 receptor antagonist [IL-1ra], and TNFα); substrate use; responses of insulin, glucose, lactate, free fatty acids (FFAs), glycerol, and cortisol to the different treatments; phosphorylation of PROs controlling PRO synthesis and degradation and postrecovery TT performance.

Subjects

Ten healthy, trained cyclists and triathletes (5 men, 5 women) between the ages of 18 and 39 years were admitted to the study. Subject characteristics are listed in Table 1. The subjects were all trained cyclists and triathletes who regularly ride distances of 60–100 miles (3.5–6 hours). The mean number of years of training and racing was 5.0 ± 1.1. Written informed consent was obtained from all subjects, and the study was approved by The University of Texas at Austin Institutional Review Board. All subjects were accustomed to cycling between 3 and 6 hours in a single ride on a regular basis. Each subject served as his or her own control

Table 1. Subject characteristics.

	Mean (10)	Men (5)	Women (5)
Age (y)	31.8 ± 1.6	32.2 ± 2.4	31.4 ± 2.5
Mass (kg)	67.8 ± 2.6	72.5 ± 3.1	63.1 ± 3.2
Height (cm)	171.1 ± 3.4	176.8 ± 3.1	165.4 ± 5.1
$\dot{V}O_2$max ($L \cdot min^{-1}$)	3.6 ± 0.2	4.2 ± 0.2	3.0 ± 0.1
$\dot{V}O_2$max ($ml \cdot kg^{-1} \cdot min^{-1}$)	52.6 ± 2.3	57.7 ± 2.8	47.6 ± 1.5

Values are mean ± *SE*.

Table 2. Energy and macronutrient composition of the recovery beverages (per 100 mL).*

	CM	CHO	PLA
CHO (g)	11.48	15.15	0
PRO (g)	3.67	0	0
Fat (g)	2.05	2.05	0
Energy (kcals)	79.05	79.05	0
Ratio of CHO:PRO	3.12:1		

*CM = chocolate milk; CHO = carbohydrate + fat; PLA = placebo; PRO = protein.

and performed the same protocol as shown in Figure 1 for each treatment.

Procedures

Experimental Beverages. The 3 treatment beverages were CM (Kirkland Organic Low-Fat CM, Costco Inc., Issaquah, WA, USA), CHO (dextrose and canola oil), or PLA (water flavored with Splenda and noncaloric Kool Aid flavoring). Energy and macronutrient composition of the beverages are shown in Table 2. The CM and CHO were isocaloric. The supplements were provided immediately postexercise after the first biopsy was performed and again 2 hours later. Subjects were instructed to drink the amount provided within 5 minutes. The amounts of supplement provided were stratified according to body weight ranges. Subjects weighing <63.6 kg (140 lb) received 500 mL per supplement (395.25 kcals each), totaling 1,000 mL and 790.5 kcals during the recovery period. Subjects weighing between 63.6 kg (140 lb) and 77.2 kg (170 lb) received 600 mL per supplement (474.3 kcals), totaling 1,200 mL and 948.6 kcals during recovery. Subjects weighing >77.2 kg (170 lb) received 700 mL per supplement (553.35 kcals), totaling 1,400 mL and 1,106.7 kcals during recovery. For the CHO treatment, the amount of fat matched that of the CM as measured for the individual's weight range. Overall, the CM treatment provided an average of 1.9 g CHO, 0.6 g PRO, and 0.3 g fat per kg body weight. The CHO treatment provided an average of 2.5 g CHO and 0.3 g fat per kg body weight. These doses were based on previous findings that 1.5 g CHO per kg body weight maximized muscle glycogen storage when supplementing immediately post and 2 hours postexercise during a 4-hour recovery period (22).

Diet and Exercise. The subjects were instructed to maintain training and dietary logs for the 2 and 3 days, respectively, before the familiarization trial and to keep training and diet consistent with that recorded for the days before the remaining 3 experimental trials. The subjects provided a copy of their training and dietary logs on the day of each trial. An investigator reviewed and verified the entries in the logs with the subjects at each session to verify that compliance with the previous logs was attained. The data from the logs were entered into Nutribase Clinical Nutrition Manager 7.17 (CyberSoft, Inc., Phoenix, AZ, USA) for nutritional analysis. Diets were not standardized, because each subject served as his or her own control. All subjects complied with the diet and exercise requirements.

Blood Sampling and Analyses. Blood sampling occurred at 6 time points during the protocol as shown in Figure 1. Saline flushes occurred every 10–15 minutes during the entire protocol to keep the catheter patent. Each 6 mL of the blood sample was mixed with 0.5 mL of ethylenediaminetetraacetic acid (EDTA) (24 mg·mL^{-1}, pH 7.4), and 0.3 ml of the anticoagulated blood was transferred to another tube containing 0.6 ml 10% perchloric acid (PCA). All tubes were centrifuged at 4°C for 10 minutes at 3,000 rpm with a HS-4 rotor in a Sorvall RC6 centrifuge (Kendro Laboratory Products, Newtown, CT, USA). After centrifugation, plasma and PCA extracts were separated into aliquots for each assay and immediately frozen and stored at −80°C for later analysis. For all assays, all samples were run in duplicate. Plasma glucose was measured using a spectrophotometric Trinder reaction (no. 315, Sigma Chemical, St. Louis, MO, USA) and had a coefficient of variation (CV) of 3.7%. Plasma insulin was measured using ImmuChem™Coated Tube ^{125}I RIA Kit (MP Biomedicals, LLC, Orangeburg, NY, USA) by radioimmunoassay with a CV of 6.0%. Blood lactate was determined from the PCA extract by enzymatic spectrophotometric analysis method based on the oxidation of lactate to pyruvate by nicotinamide adenine dicnucleotide (NAD^+) (18) and had a CV of 1.5%. Plasma FFAs were measured using the colorimetric assay procedure of Duncombe (13) but modified by using the extraction reagent of Noma et al. (36) and the copper reagent of Laurell and Tibbling (25). The CV for this assay was 5.3%. Plasma glycerol was measured from the PCA extract according to the protocol of Weiland (56). Plasma myoglobin concentrations were determined by solid phase enzyme-linked immunosorbent assay (BioCheck, Inc., Foster City, CA, USA), with a CV of 5.4%. Plasma CPK was determined spectrophotometrically using the Creatine Kinase Reagent Kit (Pointe Scientific, Inc., Canton, MI, USA). The CV for this assay was 1.0%. Plasma cortisol was measured using ImmuChem™ Coated Tube ^{125}I RIA Kit (MP Biomedicals, LLC, Orangeburg, NY, USA) by radioimmunoassay. The CV for this assay was 6.1%. Total plasma concentrations of L-1Ra, IL-6, IL-8, IL-10, and tumor necrosis factor alpha (TNFα) were determined using Millipore High Sensitivity Multiplex Human Cytokine Assay kits (Millipore, Billerica, MA, USA) with a Bio-Plex 200 multiplex suspension array system with Luminex xMAP detection technology (Luminex Corp., Austin, TX, USA).

Substrate Oxidation. Substrate use during the recovery period was estimated from the periodic measurements of RER, V_{CO_2} and $\dot{V}_{O_2}$ (minutes 30–40, 90–100, 150–160, and 210–220 of the recovery period, as shown in Figure 1). Fat and CHO oxidation rates were calculated from the $\dot{V}_{CO_2}$ and $\dot{V}_{O_2}$ values

using the equation of Frayn (15). Energy expenditure (kcal·min^{-1}) from CHO and fat was calculated from the oxidation rates by applying the Atwater general conversion factors for CHO and fat (2).

Heart Rate and Rating of Perceived Exertion. Heart rate was recorded using Polar heart rate monitors at the beginning of each exercise bout, and every 30 minutes during exercise, and every 30 minutes during the recovery phase. Subjective rating of perceived exertion (RPE) ratings on a Borg scale (ranging from 6 to 20) were obtained during exercise at the same time points as heart rate during exercise bouts.

Muscle Biopsy Procedure. Before each biopsy, the subject's thigh was cleansed with 10% betadine solution, and 1.4 mL of 1% Lidocaine Hydrochloride (Elkins-Sinn, Inc., Cherry Hill, NJ, USA) was injected. The first biopsy of each trial (R0) was taken from the vastus lateralis through a 5- to 8-mm incision made through the skin and fascia, 6 in. from the midline of the thigh on the lateral side and 2.5 in. above the patella. The second biopsy (R45) was taken from the same incision as that of the R0 sample. A new incision was made for the third biopsy (R End), 1 in. above the previous incision. The same leg was used for the first and third experimental trials, with the other leg biopsied in the second trial. The incisions for the third trial were made 1 in. above and proximal to the incisions from the first trial. Approximately ~45- to 60-mg wet weight of tissue was taken during each biopsy. The tissue samples were trimmed of adipose and connective tissue and immediately frozen in liquid nitrogen at −80°C for subsequent analysis.

Muscle Tissue Processing. The muscle samples were weighed and homogenized in ice-cold buffer containing 20 mM Hepes, 2 mM ethylene glycol tetracetic acid (EGTA), 50 mM sodium fluoride, 100 mM potassium chloride, 0.2 mM EDTA, 50 mM glycerophosphate, 1 mM dithiothreitol (DTT), 0.1 mM PMST, 1 mM benzamidine, and 0.5 mM sodium vanadate (pH 7.4) at a dilution of 1:100. Homogenization was performed on ice using Caframo RZRl Stirrer (Caframo Limited, Warton, Ontario, Canada). Half of the homogenate was used immediately for the determination of muscle glycogen concentration. The other half of the homogenate was immediately centrifuged at 14,000*g* for 10 minutes at 4°C, and the supernatant aliquoted to several test tubes and stored at −80°C for later signaling PRO analysis. Phosphorylation of mTOR, rpS6, eIF2Bε, and FOXO3A, total ubiquitination, and total α-tubulin was determined by Western blotting.

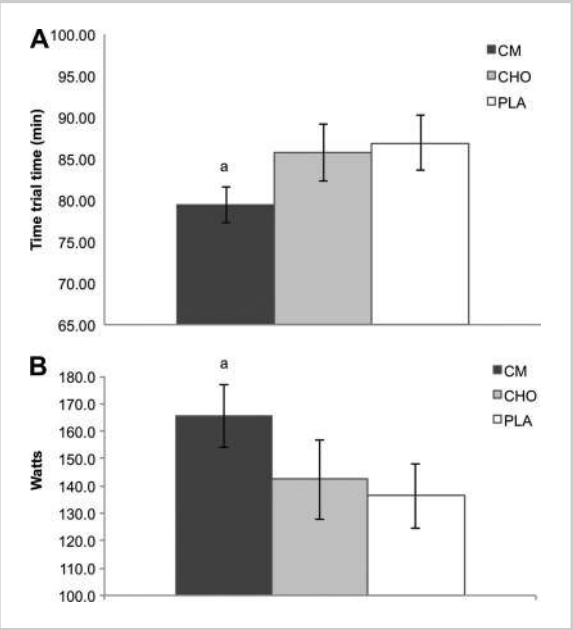

Figure 2. Time trial performance. (A) Average time to complete a 40-km cycling time trial. (B) Average watts during time trial. Values are mean ± *SE*. Significant treatment differences: [a]chocolate milk (CM) vs. carbohydrate (CHO) and placebo (PLA) ($p \leq 0.05$).

Muscle Glycogen Analysis. Muscle glycogen concentrations were determined from the freshly homogenized muscle

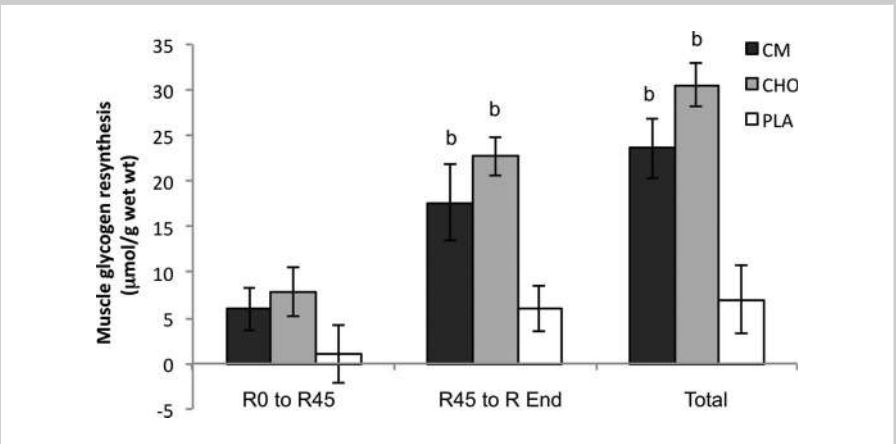

Figure 3. Muscle glycogen resynthesis during 4-hour recovery. Biopsies were taken immediately (R0), 45 minutes (R45), and 4-hour postexercise (R End). Doses of supplement were provided immediately after the biopsy occurred at R0, and at 2 hours into recovery. Resyntheses that occurred between R0 and R45 and R45 to R End are shown, and the total resynthesis over the 4-hour period (R0 to R End). Values are mean ± *SE*. Significant treatment differences: [b]chocolate milk (CM) and carbohydrate (CHO) vs. placebo (PLA) ($p \leq 0.05$).

samples after the complete enzymatic degradation from glycogen to glucose with amyloglucosidase (38). Liberated glucose was then measured using a spectrophotometric Trinder reaction (no. 315, Sigma Chemical, St. Louis, MO, USA).

Muscle Intracellular Signaling Protein Analyses. Using the supernatant aliquoted for use in signaling PRO analysis, PRO concentration was determined using a modified Lowry assay (30). Aliquots of homogenized muscle sample supernatants and standards were slowly thawed over ice and diluted 1:1 with sample buffer containing 1.25 M Tris, pH 6.8, glycerol, 20% sodium dodecyl sulfate (SDS), 2-mercaptoethanol, 0.25% bromophenol blue solution, and deionized water. Three gels were electrophoresed for each subject's 9 samples: one for detection of p-mTOR and p-FOXO3A, one for p-eIF2Bε and p-rpS6, and one for ubiquitination. Total α-tubulin, measured as a housekeeping PRO, was also detected on each gel.

Samples containing 60 μg of total PRO were separated on 10% polyacrylamide gels SDS–polyacrylamide gel electrophoresis for 48 minutes (eIF2Bε and rpS6), 104 minutes (mTOR and FOXO3A), or 45 minutes (ubiquitination) at 200 V (Bio-Rad Laboratories, Hercules, CA, USA) After electrophoresis, the gels were electrotransferred using 25 V for 15 minutes (eIF2Bε, rpS6, and ubiquitination) or 22 minutes (mTOR and FOXO3A) to 0.4 μm polyvinylidine fluoride membranes (Millipore, Bedford, MA, USA). The membranes were blocked in TTBS (TBS, 50 mM Tris, 150 mM NaCl, containing 0.06% Tween-20), and 6% nonfat dry milk (or 5% bovine serum albumin (BSA) in the case of eIF2Bε) for 1 hour at room temperature on a rocking platform at medium speed. The membranes were then washed in 1× TTBS 3 times for 5 minutes each wash. Using the molecular weight markers visible on the membranes in 3 lanes as a guide, the membrane for mTOR (~289 kD), FOXO3A (~97 kD), and α-tubulin (~55 kD) was cut into 3 sections at the 150- and 75-kD markers. The membranes for eIF2Bε (~82 kD), rpS6 (~32 kD), and α-tubulin (~55 kD) were cut into 3 sections at the 75- and 50-kD markers. The membrane for ubiquitination was not cut. (After probing for ubiquitination, the membrane

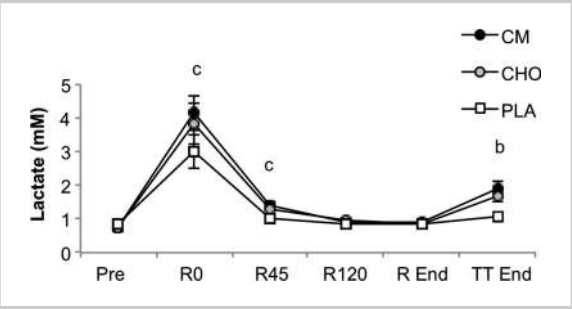

Figure 5. Blood lactate. Values are mean ± *SE*. Significant treatment differences were found for chocolate milk (CM) vs. placebo (PLA) ($p \leq 0.05$). Significant treatment by time differences: [b]CM and carbohydrate (CHO) vs. PLA; [c]CM vs. PLA ($p \leq 0.05$).

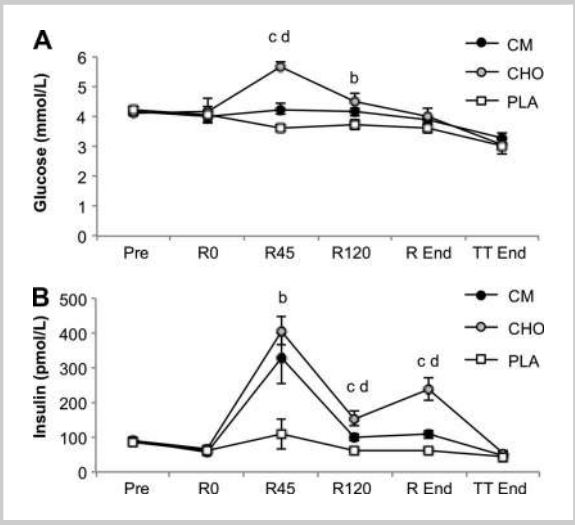

Figure 4. Plasma glucose and insulin. (A) Plasma glucose measured at 6 time points throughout the exercise, recovery, and time trial protocol. Significant treatment differences were found for carbohydrate (CHO) vs. chocolate milk (CM) and placebo (PLA), and CM vs PLA ($p \leq 0.05$). (B) Plasma insulin measured at the same time points throughout the protocol. Significant treatment differences were found for CHO vs. CM and PLA and for CM vs. PLA ($p \leq 0.05$). Values are mean ± *SE*. Significant treatment by time differences: [b]CM and CHO vs. PLA; [c]CM vs. PLA; [d]CHO vs. CM and PLA ($p \leq 0.05$).

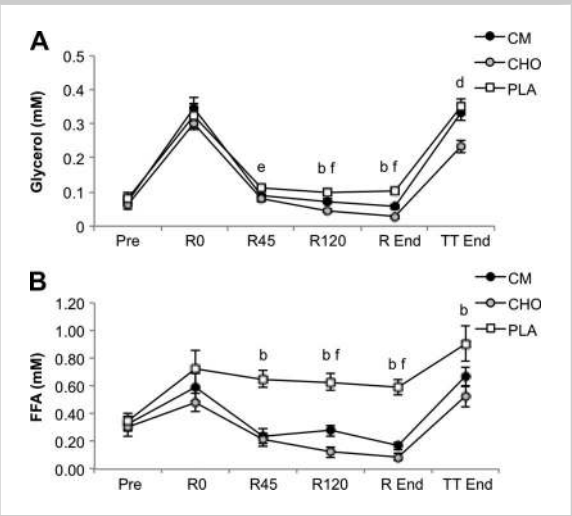

Figure 6. (A) Plasma glycerol and (B) plasma-free fatty acids. Values are mean ± *SE*. Significant treatment differences were found for placebo (PLA) vs. chocolate milk (CM) and carbohydrate (CHO) and for CM vs. CHO ($p \leq 0.05$). Significant treatment by time differences: [b]CM and CHO vs. PLA; [d]CHO vs. CM and PLA; [e]CHO vs. PLA; [f]CM vs. CHO ($p \leq 0.05$).

Table 3. Plasma markers of muscle damage and inflammation.*†

	Pre	R0	R45	R120	R End	TT End
Myoglobin (nmol·L^{-1})						
CM	1.26 ± 0.11	1.44 ± 0.06	2.16 ± 0.32		1.53 ± 0.16	1.87 ± 0.14‡
CHO	1.26 ± 0.12	1.45 ± 0.11	2.01 ± 0.36		1.91 ± 0.35	2.16 ± 0.32‡
PLA	1.35 ± 0.13	1.68 ± 0.20	2.37 ± 0.42		1.73 ± 0.10	1.99 ± 0.21‡
CPK (U·L^{-1})						
CM	125.80 ± 27.42	157.97 ± 38.30		146.69 ± 46.22	161.85 ± 45.96	206.03 ± 64.97‡
CHO	94.56 ± 22.85	114.34 ± 25.81		107.50 ± 23.20	124.78 ± 35.97	167.19 ± 35.53‡
PLA	179.25 ± 76.91	207.82 ± 86.59		178.79 ± 62.46	207.69 ± 69.82	240.39 ± 69.75‡
Cortisol (pmol·L^{-1})						
CM	729.1 ± 96.7	837.4 ± 62.1	595.3 ± 61.0	433.9 ± 69.7	382.8 ± 57.5	902.1 ± 69.1‡
CHO	698.9 ± 70.0	787.4 ± 119.7	668.4 ± 82.8	539.2 ± 80.1	414.7 ± 53.9	1154.8 ± 143.0‡
PLA	720.2 ± 84.2	894.5 ± 133.3	606.3 ± 73.0	491.9 ± 75.4	370.0 ± 44.8	874.7 ± 103.3‡
IL-6 (pg·mL^{-1})						
CM	38.48 ± 17.01	62.18 ± 21.29	40.24 ± 15.95	57.32 ± 18.38	47.16 ± 15.24	97.39 ± 33.02‡
CHO	55.00 ± 20.14	93.01 ± 38.17	58.49 ± 19.23	79.93 ± 23.39	67.01 ± 19.53	97.16 ± 38.56‡
PLA	60.17 ± 31.19	109.79 ± 52.59	85.01 ± 38.69	81.86 ± 22.47	81.00 ± 27.10	139.72 ± 57.34‡
IL-8 (pg·mL^{-1})						
CM	24.51 ± 4.73	38.38 ± 8.26	28.69 ± 5.35	30.35 ± 5.84	26.69 ± 5.21	39.02 ± 8.63‡
CHO	29.12 ± 7.18	42.22 ± 9.86	31.65 ± 7.31	29.50 ± 6.32	24.97 ± 5.29	40.82 ± 7.37‡
PLA	29.16 ± 6.79	41.68 ± 9.90	37.64 ± 9.04	34.76 ± 8.34	34.21 ± 8.53	40.52 ± 17.14‡
IL-10 (pg·mL^{-1})						
CM	133.78 ± 87.28	213.63 ± 114.98	174.48 ± 99.87	157.20 ± 78.88	140.04 ± 71.90	286.63 ± 135.93
CHO	124.18 ± 59.86	258.56 ± 130.82	225.11 ± 116.35	237.41 ± 119.95	187.66 ± 86.92	219.92 ± 92.27
PLA	162.30 ± 101.89	264.18 ± 159.08	188.65 ± 88.52	229.49 ± 115.90	206.59 ± 111.96	367.82 ± 210.10
IL-1Ra (pg·mL^{-1})						
CM	67.45 ± 8.64	86.19 ± 10.07	67.21 ± 7.16	45.38 ± 11.17	55.27 ± 9.40	54.31 ± 9.74‡
CHO	69.83 ± 12.84	78.54 ± 10.49	66.38 ± 8.76	36.32 ± 6.41	47.97 ± 8.72	56.33 ± 7.07‡
PLA	65.31 ± 7.60	79.11 ± 6.55	59.52 ± 9.09	49.93 ± 6.29	63.66 ± 7.54	58.15 ± 8.90‡
TNF-α (pg·mL^{-1})						
CM	8.21 ± 1.86	9.23 ± 1.64	6.22 ± 1.16	5.10 ± 1.07	6.43 ± 1.43	6.95 ± 1.83‡
CHO	9.51 ± 2.81	9.45 ± 1.83	5.82 ± 0.77	5.20 ± 0.88	5.12 ± 1.19	6.48 ± 1.36‡
PLA	8.15 ± 1.65	9.12 ± 1.95	7.13 ± 1.46	5.51 ± 1.22	6.62 ± 1.57	6.76 ± 1.22‡

*CM = chocolate milk; CHO = carbohydrate + fat; PLA = placebo; PRO = protein; CPK = creatine phosphokinase; TNF = tumor necrosis factor; R0 = recovery minute 0; R45 = recovery minutes 45; R120 = recovery minutes 120; RR End = ; TT = time trial.

†Values are mean ± *SE*.

‡Significant change over time only, $p < 0.05$.

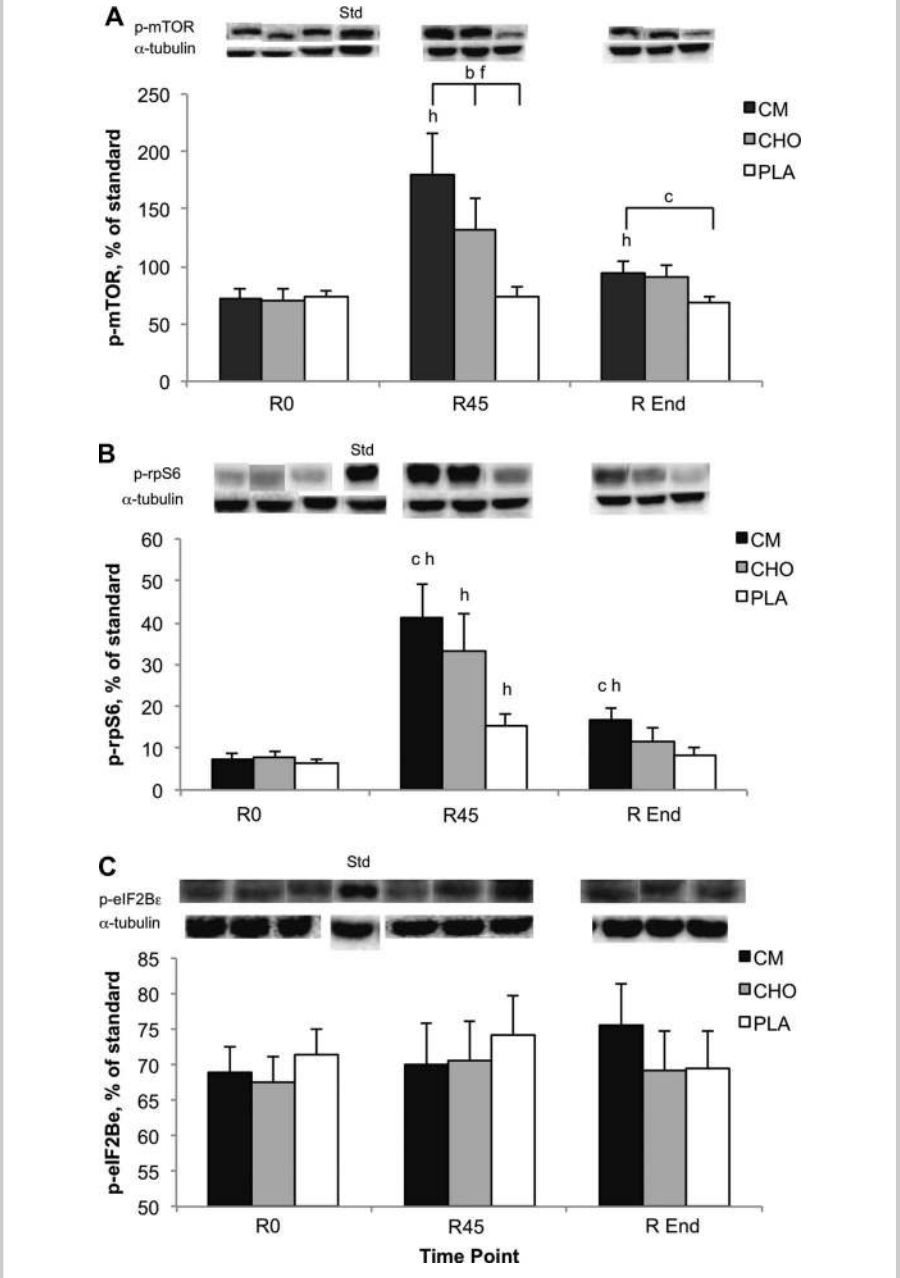

Figure 7. (A) Mammalian target of rapamycin (mTOR) phosphorylation (Ser2448). A significant overall treatment difference existed between chocolate milk (CM) and placebo (PLA) ($p \leq 0.05$). Significant treatment by time differences: [b]CM vs. carbohydrate (CHO) and PLA; [c]CM vs. PLA; [f]CM vs. CHO; [h]within-treatment significant difference from R0 ($p \leq 0.05$). (B) rpS6 (Ser 235/236) phosphorylation. A significant overall treatment difference existed between CM vs. CHO and PLA ($p \leq 0.05$). Significant treatment by time differences: [c]CM vs. PLA; [h]within-treatment significant difference from R0 ($p \leq 0.05$). (C) eIF2Bε (Ser539) phosphorylation. No significant treatment, time, or treatment × time differences were found.

section was stripped, reblocked in 6% non-fat dry milk (NFDM)–TTBS, and reprobed for α-tubulin.)

Each membrane or section of membrane was incubated overnight at 4°C on a rocking platform at low speed with antibodies directed against p-mTOR (Ser2448, no. 2971S, Cell Signaling, Danvers, MA, USA), p-rpS6 (Ser235/236, no. 9205S, Cell Signaling), p-eIF2Bε (Ser539, no. PS1017, EMD Calbiotech/Merck KGaA, Darmstadt, Germany), p-FOXO3A (Ser318/321, no. 9465, Cell Signaling), ubiquitin (no. 9133, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and α-tubulin (no. 2144, Cell Signaling). The antibodies were diluted 1:800 (mTOR), 1:900 (α-tubulin), 1:500 (FOXO3A and ubiquitin), and 1:1,000 (rpS6 and eIF2Bε) in TTBS containing 2% nonfat dry milk for mTOR, α-tubulin, and ubiquitin; for FOXO3A and eIF2Bε, primary incubation was done in TTBS with 5 and 1% BSA (no. 105033, ICN Biochemicals, Costa Mesa, CA, USA), respectively. After the overnight incubation, membranes were washed 3 times with TTBS for 5 minutes each wash and incubated for 1.5 hours for mTOR, eIF2Bε, and FOXO3A and 2 hours for α-tubulin and ubiquitin) with a secondary antibody (goat antirabbit, horseradish peroxides–linked IgG, no. 7074, Cell Signaling). Dilutions were 1:800 (mTOR), 1:1,000 (α-tubulin), 1:1,500 (FOXO3A), and 1:2,000 (rpS6) in TTBS containing 2% nonfat dry milk. For eIF2Bε, secondary incubation was in TTBS with 1% BSA (no. 105033, ICN Biochemicals, Costa Mesa, CA.) at a dilution of 1:3,000. The antibody-bound PROs on the immunoblots were visualized by enhanced chemiluminescence according to the manufacturer's protocol (Perkin Elmer, Boston, MA, USA) using a Bio-Rad ChemiDoc detection system, and the mean density of each band was quantified using Quantity One 1-D Analysis software (Bio-Rad Laboratories, Hercules, CA, USA). A molecular weight ladder (Precision Plus Protein

Standard, Bio-Rad) and a rodent internal control standard prepared from insulin-stimulated mixed skeletal muscle were also included on each gel. All blots were compared with the rodent control standard, and the values of each sample were represented as a percent of standard for each blot.

Statistical Analyses

Plasma insulin, glucose, lactate, myoglobin, CPK, FFA, glycerol, cortisol, and cytokines were analyzed using 2-way (treatment × time) analysis of variance (ANOVA) for repeated measures. Time trial measures (TT time, average power output, average heart rate, and average RPE) were analyzed using 1-way ANOVA for repeated measures. Differences in muscle glycogen resynthesis from R0 to R45, R45 to R End and Total resynthesis (R0 to R End) were analyzed using a 2-way (treatment × time) ANOVA for repeated measures. Signaling PRO phosphorylation or content was analyzed using 2-way ANOVAs for repeated measures (treatment × time and time × treatment). Substrate oxidation and energy expenditure during the recovery period were analyzed using 2-way (treatment × time) ANOVA for repeated measures. For all measures, post hoc analysis was performed when significance was found using least significant difference. Differences were considered significant at $p \leq 0.05$. Data were expressed as mean ± *SE*. All statistical analyses were performed using SPSS version 16.0 statistical software (SPSS Inc., Chicago, IL, USA).

Results

Time Trial Performance

Time trial time (TTT) is shown in Figure 2A. The TTT was significantly shorter in CM compared to in both CHO and PLA ($p \leq 0.05$). Average power output (Watts) was significantly higher in CM compared to in both CHO and PLA (Figure 2B; $p \leq 0.05$). Heart rate during the TT was significantly higher in CM than in CHO or in PLA (162.8 ± 5.6 vs. 145.9 ± 7.4 and 148.9 ± 7.1, respectively, $p \leq 0.05$), whereas RPE was not significantly different between the 3 treatments (14.5 ± 0.5 vs. 14.0 ± 0.5 and 14.0 ± 0.5, respectively, $p \leq 0.05$). There was no gender effect relative to the effect of supplement on performance.

Muscle Glycogen

Muscle glycogen resynthesis values are shown in Figure 3. Total muscle glycogen resynthesis over the 4-hour recovery period was significantly greater in both the CM and CHO treatments than in PLA (23.58 and 30.58 $\mu mol \cdot g^{-1}$ wet weight, respectively, vs. 7.05 $\mu mol \cdot g^{-1}$ wet weight, $p \leq 0.05$). The CHO was not significantly different from CM, although significance was approached ($p = 0.06$).

Blood and Plasma Analyses

A significant treatment effect existed for plasma glucose in CHO compared to in CM and in PLA, and in CM compared to in PLA (Figure 4A; $p \leq 0.05$). At R45, plasma glucose in CHO was significantly higher than CM and PLA, and CM was significantly higher than PLA ($p \leq 0.05$). At R120, both CHO and CM were higher than PLA ($p \leq 0.05$), with no significant differences between CHO and CM. At R End and TT End, no differences existed between the 3 treatments (Figure 4A). Plasma insulin (Figure 4B) was also significantly higher in the CHO treatment compared to in CM and PLA and CM compared to in PLA ($p \leq 0.05$). Both CHO and CM were significantly higher than PLA at R45 ($p \leq 0.05$). At R120 and R End, plasma insulin levels in CM had decreased to near baseline levels but were still significantly higher than in PLA ($p \leq 0.05$). However, plasma insulin in CHO was significantly higher at R120 and R End than in CM and PLA. At the TT End, no treatment differences were detected (Figure 4B; $p \leq 0.05$).

A significant overall treatment effect for blood lactate was observed between CM and PLA ($p \leq 0.05$). As shown in Figure 5, blood lactate levels rose significantly during the first exercise bout (Pre to R0) in all treatment groups ($p \leq 0.05$), although a slight but significantly lower blood lactate level was found in the PLA treatment at R0 and R45 compared to in the CM ($P \leq 0.05$). No differences existed between the 3 treatments at R120 and R End. At TT End, blood lactate

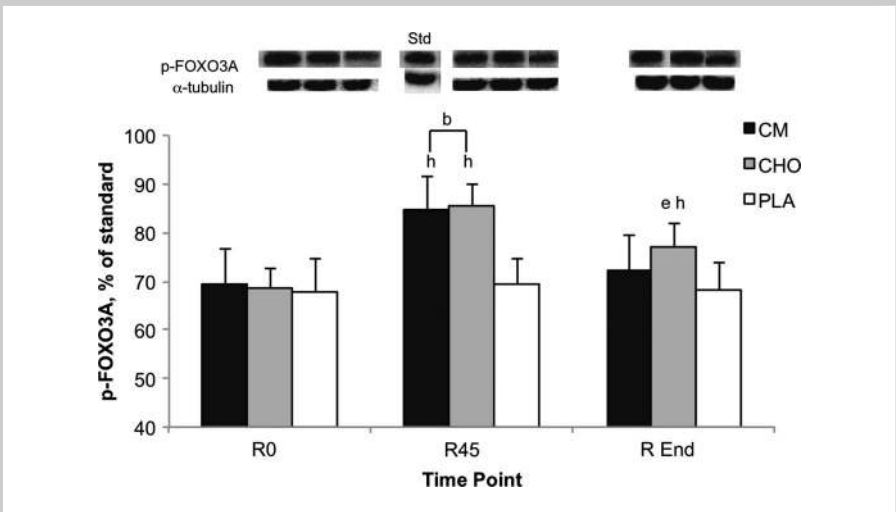

Figure 8. FOXO3A (Ser318/321) phosphorylation. A significant overall treatment difference existed between carbohydrate (CHO) and placebo (PLA) ($p \leq 0.05$). Significant treatment by time differences: [b]chocolate milk (CM) and CHO vs. PLA; [e]CHO vs. PLA; [h]within-treatment significant difference from R0 ($p \leq 0.05$).

Table 4. Average substrate use during 4-hour recovery period.*†‡

	CM	CHO	PLA
CHO oxidation ($g \cdot min^{-1}$)	0.159 ± 0.023	0.183 ± 0.028	0.079 ± 0.024§
Fat oxidation ($g \cdot min^{-1}$)	0.093 ± 0.009	0.066 ± 0.010‖	0.102 ± 0.010
CHO EE ($kcals \cdot min^{-1}$)	0.669 ± 0.096	0.770 ± 0.119	0.332 ± 0.099§
Fat EE ($kcals \cdot min^{-1}$)	0.843 ± 0.078	0.599 ± 0.093‖	0.926 ± 0.090

*CM = chocolate milk; CHO = carbohydrate + fat; PLA = placebo; PRO = protein; EE = energy expenditure.
†Values are mean ± *SE*.
‡Average of four 10-minute gas collections (minutes 30, 90, 150, and 210) of recovery period.
§PLA vs. CM and CHO, $p < 0.05$.
‖CHO vs. CM and PLA, $p < 0.05$

was significantly higher in CM and in CHO than in PLA ($p \leq 0.05$). For both plasma glycerol and FFAs, significant treatment effects were found in PLA compared to in CHO and in CM ($p \leq 0.05$) and between CM and CHO (Figure 6A and B; $p \leq 0.05$). At R45, plasma glycerol was significantly higher in PLA than in CM only, and FFAs were significantly higher in PLA than in both CM and CHO ($p \leq 0.05$). At R120 and R End, plasma glycerol and FFAs were significantly higher in PLA than in CHO and in CM, and in CM compared to in CHO ($p \leq 0.05$). At TT End, plasma glycerol in CHO was significantly lower than in CM and in PLA ($p \leq 0.05$), and plasma FFAs were higher in PLA compared to in both CHO and CM at TT End ($p \leq 0.05$), suggesting that more fat was available to be used as fuel in the PLA and CM treatments compared to in the CHO treatment.

As is shown in Table 3, plasma myoglobin, CPK, cortisol, and cytokines IL-6, IL-8, IL-1Ra, and TNFα rose significantly over time in all treatments ($p \leq 0.05$), with no significant differences found between the treatments. The increase in all of these analytes from Pre to R0, and in blood lactate, indicates that the subjects were cycling at a high intensity during the initial bout. All decreased from the R0 values during the 4-hour recovery period, and increased at TT End, with no significant treatment differences detected.

Intracellular Signaling Proteins

Significant differences in mTOR phosphorylation are shown in Figure 7A. A significant overall treatment difference was found for mTOR phosphorylation between CM and PLA ($p \leq 0.05$). No difference in phosphorylation status of mTOR was detected between treatments at R0. However, at 45 minutes of recovery (R45), mTOR phosphorylation was significantly greater in the CM treatment compared to in the CHO and PLA treatments (Figure 7A). At the end of recovery (R End), phosphorylation was greater in CM compared to PLA ($p \leq 0.05$). At R45, phosphorylation in both CM and CHO was significantly greater than at R0 for the respective treatments, and at R End, this elevated phosphorylation status persisted only in CM (Figure 7A; $p \leq 0.05$).

The phosphorylation pattern of rpS6 (Figure 7B) was similar to that of mTOR, with a significant overall treatment difference between CM vs. CHO and PLA ($p \leq 0.05$). At R0, no differences in rpS6 phosphorylation were detected between treatments. At R45, phosphorylation of rpS6 was increased significantly, with the increase in CM significantly greater than in CHO and PLA ($p \leq 0.05$). At R End, phosphorylation in CM remained elevated compared to the R0 value and was significantly greater than CHO and PLA ($p \leq 0.05$), whereas phosphorylation in CHO and in PLA had returned to levels that were not significantly greater than their respective R0 values (Figure 7B).

FOXO3A phosphorylation is shown in Figure 8. A significant overall treatment difference existed for FOXO3A phosphorylation between CHO and PLA ($p \leq 0.05$). At R0, no differences in FOXO3A phosphorylation existed between treatments. At R45, FOXO3A phosphorylation was significantly greater in CHO and CM compared to in PLA, and compared to the respective R0 levels (all $p \leq 0.05$). By R End, phosphorylation remained elevated only in CHO compared to in CM and PLA, and compared to the R0 level (Figure 8, $p \leq 0.05$).

No differences were detected for p-eIF2Bε (Figure 7C) or total ubiquitination (data not shown) for treatment, time or treatment × time interactions.

Substrate Use during Recovery

Carbohydrate and fat energy expenditure and CHO and fat oxidation rates during the recovery period are shown in Table 4. Average CHO energy expenditure ($kcal \cdot min^{-1}$) and CHO oxidation ($g \cdot min^{-1}$) were significantly lower in PLA than in CM or in CHO ($p \leq 0.05$). Average fat energy expenditure ($kcal \cdot min^{-1}$) and fat oxidation ($g \cdot min^{-1}$) were significantly lower in the CHO treatment than in CM or in PLA (both $p \leq 0.05$).

Discussion

This study aimed to characterize the roles of an initial bout of strenuous exercise and nutritional supplementation on subsequent exercise performance and key aspects of exercise recovery, namely, muscle glycogen resynthesis, markers of muscle damage and inflammation, and activation states of intramuscular signaling PROs involved in PRO synthesis and degradation. The key finding of this study is that a popular dairy beverage containing both CHO and PRO (low fat CM) was more effective in improving cycling TT performance in a subsequent bout compared to an isocaloric CHO or a PLA supplement. This performance improvement occurred despite there being no increase in the subjective RPEs, suggesting that they did not perceive their effort as more difficult although they were able to exercise at a higher intensity with the CM treatment compared to CHO and PLA. Furthermore, we report here that CM increased the activation status of signaling PROs associated with increased mRNA translation and PRO synthesis compared to PLA. These findings are novel because the recovery supplement used in comparison to CHO and PLA is a commonly available, organic dairy beverage, CM, rather than a CHO + PRO supplement commercially designed for exercise recovery. In addition, the present investigation examines many aspects of the recovery process with the aim of addressing possible mechanisms of improved recovery and performance.

The finding of improved subsequent performance is in agreement with several other studies that reported improved endurance performance in a subsequent bout when ingesting a CHO + PRO supplement compared to CHO alone (3,10,35,46,57), and with those of investigations comparing CM to other CHO + PRO supplements (40) and to both CHO + PRO and CHO only supplements (50). However, not all investigations report improvements in subsequent performance with CHO + PRO supplementation compared to CHO (7,8,43–45). Differences in protocol design may lead to the inconsistent findings across investigations of CHO + PRO (including CM) supplementation. In some studies, supplements were given during the initial exercise bout (7,8) or both during and postexercise (46), whereas in this study and others, the supplements were given immediately postexercise and again during the recovery period (35,57). The timing of the subsequent exercise trials also varied between studies, including ~15 hours later (46,40), 22–24 hours later (7,8,43–45,37) 1 and 2 days after the initial bout (45) or after a 3- to 4-hour recovery, as in this study (40,50,57). Differences also exist for the type of exercise trial employed to assess subsequent performance and recovery: TTT (7,8), TTE (37,43,44,46), or repeated sprint performance (45). Therefore, these and other methodological differences must be considered when interpreting the results of CHO + PRO vs. CHO investigations.

One proposed mechanism for improved performance after recovery with CHO + PRO supplementation is increased muscle glycogen resynthesis during the recovery period. Several investigations have shown that CHO + PRO supplementation can increase the rate of muscle glycogen synthesis beyond that of CHO alone (4,21,52,57,59) and therefore may contribute to improved exercise performance in a subsequent bout. In this study, we did not find a significant difference between CM and CHO in muscle glycogen resynthesis, although both were significantly greater than in PLA. However, only in the CM treatment was TT performance significantly improved compared to CHO and PLA. Interestingly, although CHO demonstrated a slight, nonsignificantly higher ($p = 0.06$) total muscle glycogen resynthesis compared to CM, this did not result in improved TT performance in CHO compared to in PLA. Therefore, the data suggest that muscle glycogen synthesis is not the mechanism through which subsequent performance was improved in this study. Possible reasons for the lack of a relationship between TT performance and muscle glycogen resynthesis are that muscle and liver glycogen levels were perhaps not sufficiently depleted in the initial bout, and the length of the subsequent bout (40 km) may not have been long enough for muscle glycogen to be a rate-limiting factor in performance.

The lack of a significant difference in muscle glycogen resynthesis between CM and CHO may be because of the observed difference in the insulin response. Although we found a lower plasma insulin response with CM compared to with CHO in this study, previous investigations have shown higher insulin responses with CHO +PRO compared to with CHO (48,57,59). However, most prior investigations of CHO + PRO supplementation have used a whey PRO-containing CHO + PRO beverage rather than CM. It is likely that the different osmolarity and gastric emptying rates of CM compared to those of CHO could have slowed down the intestinal transport of CHOs and AAs, resulting in a lower plasma glucose and insulin response, and their subsequent availability to the muscle, in CM compared to in CHO. This likely explains the lack of a significant difference in muscle glycogen resynthesis between these 2 nutrient-containing treatments. In addition, CM contains many micronutrients that may not be found in other CHO + PRO supplements, and the effects of these components on the measures investigated here are unknown at this time.

Another proposed mechanism for subsequent performance improvement with a CHO + PRO–containing supplement is muscle damage attenuation. Saunders et al. (46) demonstrated that CHO + PRO supplementation improved recovery from an initial strenuous exercise bout such that subsequent exercise performance was improved and noted that muscle damage marker CPK was suppressed in the CHO + PRO treatment. In this study, however, this association between improved subsequent performance and reduced muscle damage indicators myoglobin and CPK was not demonstrated. In addition, we did not detect treatment differences in any of the pro or anti-inflammatory

cytokines (Table 3). Taken together, our findings suggest that neither reduced muscle damage nor a reduced inflammatory response during the recovery period underlies the subsequent performance differences 4 hours later.

In addition to plasma markers of muscle damage and inflammation, we assessed 2 {PRO in the skeletal muscle samples that regulate PRO degradation–FOXO3A and ubiquitin. Protein degradation involves the ubiquitin–proteasome pathway (UPP), the primary degradation system for skeletal muscle (26,49). A key component of the UPP is ubiquitin itself. This highly conserved, 8.5-kD PRO covalently binds to abnormal or damaged PROs, targeting them to be degraded by the 26S proteasome (9,17). Recently, a few investigations have used total ubiquitin content of Western blot samples as an indicator of PRO degradation, demonstrating an increase in total ubiquitination in conditions of high proteolysis (14,39). This method was employed in this study to assess total ubiquitination in the biopsy samples during the recovery period, and despite a trend for slightly increased ubiquitination in the CHO treatment, there was not a statistically significant difference in treatment or in time between the three treatments (data not shown).

Key apoptotic genes in the UPP share a common critical transcription factor, forkhead box 3A (FOXO3A). When the insulin signaling pathway is activated, Akt is phosphorylated, translocates into the nucleus, and phosphorylates FOXO3A on serine residues including 253, 318, and 321, which causes the nuclear export of FOXO3A into the cytoplasm (19,34,47). Thus, FOXO3A is deactivated and prevented from promoting apoptosis. A few studies have verified a role for FOXO3A in proteolysis after resistance exercise (27,29,42,58) and running exercise (29). In this study, we hypothesized that we would detect greater FOXO3A phosphorylation in the CM treatment compared to CHO and PLA. FOXO3A phosphorylation increased significantly from R0 to R45 in both CM and CHO (Figure 8, $p \leq 0.05$), and although phosphorylation in both CM and CHO was significantly greater than in PLA, no difference was found between CM and CHO. At R End, FOXO3A phosphorylation remained elevated from R0 only in CHO and was significantly greater compared to PLA. Because FOXO3A is activated through the insulin signaling pathway, the increased phosphorylation of FOXO3A at R45 in CHO and CM and at R End in CHO likely demonstrates the effect of insulin. Because the insulin response in CHO at R End was higher than in CM or in PLA, it appears that this was responsible for the greater FOXO3A phosphorylation at R End in CHO compared to in PLA. However, it also appears that the small increase in phosphorylation in FOXO3A was not sufficient to limit PRO degradation in the CM and CHO treatments as verified by the markers of muscle damage assessed and the total ubiquitination results.

Our finding that degradation signaling was not significantly reduced with supplementation in this study is supported by the lack of reductions in the plasma muscle damage and inflammatory markers. Given that the CM and CHO treatments did not appear to block the processes of muscle damage and degradation, it may be that providing supplementation after the initial exercise bout, rather than both during and postexercise as has been done previously (46), minimized the effect of supplementation in these areas. Therefore, these data suggest that although attenuation of muscle damage or decreased PRO degradation during recovery from strenuous exercise may occur in response to CHO + PRO or CM supplementation, these are not the mechanisms by which TT performance was improved in this study.

Activation of PRO synthesis is an important metabolic response in the recovery process and is paramount for muscle tissue repair and training adaptation. As mentioned previously, the balance between PRO degradation and synthesis postexercise is mediated in part by the availability of nutrients, most importantly, the availability of AAs. Aminoacid infusion or ingestion has been shown to stimulate muscle PRO synthesis both at rest (5,12) and after resistance exercise (6,51), as has the combination of essential AA and CHO after resistance exercise (11,41). There is increasing evidence that the combined ingestion of CHO and either PRO or AA postexercise can have an additive effect on PRO synthesis (28,32). This is partly likely because of the synergistic effect of CHO + AA or CHO + PRO supplementation on the plasma insulin response (48,58) and the maintenance of an elevated plasma AA profile. This may lead to a greater activation of translation initiation. As the rate-limiting step in mRNA translation, translation initiation is regulated in part by the mTOR pathway. mTOR phosphorylates 2 downstream targets, including p70S6K (31). p70S6K then phosphorylates ribosomal PRO S6 (rpS6), which enhances translation of a class of mRNAs that encode ribosomal PROs, elongation factors, and binding PROs (24). This results in an increase in the capacity of the cell to synthesize PRO. The eukaryotic initiation factor 2B (eIF2B) also plays a key role in translation initiation. eIF2B catalyzes the GDP-GTP exchange on eIF2, which is essential for control of ribosomal formation and mRNA translation (23). eIF2Bε is the largest catalytic subunit of this PRO and is phosphorylated by GSK-3B at Ser 539 (54), which inhibits the guanine nucleotide exchange activity of eIF2B. Phosphorylation of GSK-3B via the insulin signaling pathway inhibits GSK-3B, releasing its inhibition of eIF2Bε.

In this study, mTOR phosphorylation increased significantly from R0 to R45 only in CM and CHO, and mTOR phosphorylation in CM was significantly greater than in CHO or in PLA. At the R End, mTOR phosphorylation in both CM and CHO was still significantly greater than in PLA, which remained unchanged throughout recovery. Only in CM was mTOR phosphorylation still significantly elevated compared to R0. rpS6 phosphorylation increased from R0 to R45 in all treatments, but only in CM was it

significantly higher than in PLA. At the R End, rpS6 phosphorylation in CM remained elevated compared to R0, and the treatment difference between CM and PLA remained significant. The increased phosphorylation of mTOR and rpS6 in CM compared to in CHO and in PLA at R45 is likely because of the combined effects of AA and insulin, whereas the increase in CHO over PLA is likely because of the effect of insulin.

Based on previous results from our laboratory that demonstrated significantly increased phosphorylation of GSK-3B with CHO + PRO supplementation postendurance exercise compared to exercise only (20), we hypothesized that we would demonstrate reduced phosphorylation of eIF2Bε, a downstream target of GSK-3B, with the CM treatment compared to CHO and PLA. However, we did not detect a significant difference in treatment or time for eIF2Bε phosphorylation in this study. This finding is supported by that of Glover et al. (16), who found that although eIF2Bε phosphorylation was significantly decreased by a strenuous resistance exercise bout compared to a baseline measure, postexercise supplementation containing CHO, PRO, and fat had no additional effect during a 6-hour recovery (16). Despite demonstrating increased activation of some of the key components of the mTOR pathway in this study, it may be that eIF2Bε phosphorylation may not be responsive to nutrient intervention, because eIF2B can be activated independent of the mTOR pathway (55). Therefore, it is possible that the effects of exercise alone are sufficient for activation of this enzyme, as demonstrated by Glover et al. (16).

The greater phosphorylation status of many of the key PROs involved in mRNA translation initiation and PRO synthesis in the present investigation is in agreement with previous findings from our laboratory. Recently, Ivy et al. (20) investigated the effects of a CHO + PRO supplement compared to PLA on intracellular signaling PROs involved in increased PRO and glycogen synthesis. Trained cyclists cycled at 75% $\dot{V}O_2$max for 45 minutes, followed by 5 1-minute sprints alternating between 90 and 45% $\dot{V}O_2$max. Immediately postexercise and again 15 minutes later, subjects ingested 400 ml of a CHO + PRO beverages (7.8% dextrose and 1.8% PRO electrolyte) or PLA. Biopsies of the vastus lateralis were taken before exercise and at 45-minute of recovery. At 45 minutes, phosphorylation of mTOR and rpS6 was increased above pre-exercise levels in the CHO + PRO treatment compared to PLA (20). The results of the present study extend these previous findings by comparing a CHO + PRO supplement, CM, to an isocaloric CHO and to PLA. Taken together, these results suggest that although providing nutritional supplementation post-exercise is more effective in increasing the stimulus for PRO synthesis and reducing PRO degradation than when postexercise nutritional intake is absent, a CHO + PRO–containing supplement such as CM is more effective overall than CHO alone in promoting an anabolic intracellular environment postexercise.

In conclusion, CM is more effective than isocaloric CHO or placebo in improving subsequent TT performance in highly trained endurance athletes. The CM is also more effective in modulating the activation of key intracellular signaling PROs involved in PRO synthesis during recovery from endurance exercise. This greater stimulus for PRO synthesis, in addition to the possible attenuation of muscle PRO degradation and faster tissue repair, during recovery may be advantageous under conditions when rapid recovery between race events or training sessions is necessary. The CM supplementation may also lead to increased training adaptations over time if the exercise and supplementation is performed on a regular basis. More research is necessary to determine the effects of CM in other models of exercise recovery, such as after resistance exercise. Finally, these findings must be considered relative to the limitations of the study including number and fitness level of the subjects tested, exercise mode, recovery time, and supplements provided.

Practical Applications

Proper nutritional supplementation is critical for facilitating the recovery and adaptation processes after strenuous endurance exercise. The present investigation demonstrates that consuming CM, an easily assessable and relatively inexpensive dairy product, after intense endurance exercise can improve performance in a cycling TT performed 4 hours after an initial, strenuous cycling ride compared to CHO only or PLA. This is an important finding for athletes who must train twice a day or compete in multiple events with limited time to recover. In addition, the potential for facilitating greater training adaptation with regular postexercise CM ingestion makes this supplementation strategy practical and attractive for both athletes and recreational exercisers.

Acknowledgments

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