Chocolate Milk and Endurance Exercise Recovery: Protein Balance, Glycogen, and Performance

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[1]Exercise Science Department, Southern Connecticut State University, New Haven, CT; [2]Department of Nutritional Sciences, University of Connecticut, Storrs, CT; [3]School of Health Sciences, Eastern Michigan University, Ypsilanti, MI; and [4]Department of Kinesiology, University of Connecticut, Storrs, CT

ABSTRACT

LUNN, W. R., S. M. PASIAKOS, M. R. COLLETTO, K. E. KARFONTA, J. W. CARBONE, J. M. ANDERSON, and N. R. RODRIGUEZ. Chocolate Milk and Endurance Exercise Recovery: Protein Balance, Glycogen, and Performance. *Med. Sci. Sports Exerc.*, Vol. 44, No. 4, pp. 682–691, 2012. **Purpose**: This study examined effects of fat-free chocolate milk (MILK) consumption on kinetic and cellular markers of protein turnover, muscle glycogen, and performance during recovery from endurance exercise. **Methods**: Male runners participated in two trials separated by 1 wk and consumed either MILK or a nonnitrogenous isocaloric carbohydrate (CHO) control beverage (CON) after a 45-min run at 65% of $\dot{V}O_{2peak}$. Postexercise muscle protein fractional synthetic rate (FSR) and whole-body protein turnover were determined during 3 h of recovery using muscle biopsies and primed constant infusions of L-[*ring*-$^2H_5$]phenylalanine and L-[1-^{13}C]leucine, respectively. Phosphorylation of translational signaling proteins and activity of proteolytic molecules were determined using Western blotting and enzymatic activity assays. Muscle glycogen was quantified, and treadmill time to exhaustion was determined after the recovery period. **Results**: Consuming MILK after exercise resulted in higher mixed muscle FSR with lower whole-body proteolysis and synthesis compared with CON ($P \leq 0.05$). Phosphorylation of eIF4E-BP1 and FOXO3a was higher for MILK ($P < 0.01$), whereas Akt phosphorylation was lower during recovery regardless of dietary treatment ($P < 0.05$). Enzymatic activity assays indicated lower caspase-3 activity during recovery for MILK ($P < 0.01$) and higher 26S proteasome activity for CON ($P < 0.01$). Muscle glycogen was not affected by either dietary treatment; however, time to exhaustion was greater for MILK than for CON ($P < 0.05$). **Conclusions**: The effects of consumption of MILK after endurance exercise on FSR, signaling molecules of skeletal muscle protein turnover, leucine kinetics, and performance measures suggest unique benefits of milk compared with a CHO-only beverage. **Key Words:** PROTEIN SYNTHESIS, INTRACELLULAR SIGNALING, PROTEOLYSIS, LEUCINE KINETICS, TIME TO EXHAUSTION, MUSCLE GLYCOGEN

Numerous researchers have investigated muscle glycogen repletion as a primary outcome of carbohydrate (CHO)-only or combined protein–CHO nutrition interventions during recovery from endurance exercise (14,16,34,37,38). Endurance athletes are challenged to recover, in the context of body protein, on the basis of the demands of their activity. Largely neglected end points in these feeding studies, however, are indices of protein turnover.

Protein turnover is defined by the balance between catabolism, or breakdown, and anabolism, or synthesis. The catabolic nature of an acute endurance exercise bout has been documented in the literature by enhanced messenger RNA expression or activity of ubiquitin–proteasome pathway–related proteins such as FOXO3a, a unique transcription factor that regulates multiple cellular processes, including the transcription of some atrophy-related genes (10,21), and the cysteine protease caspase-3 (19) in the postexercise recovery period. Empirical evidence of reduced activity of proteolytic biomarkers after amino acid provision, however, is limited to rodent models (4,5). Regarding anabolism, provision of protein–CHO nutrition after exercise has been shown to augment the increase in fractional synthetic rate (FSR) of mixed skeletal muscle protein in the postabsorptive state, but data from an endurance mode are lacking (12). Evidence of activation of translational signaling molecules in the Akt–mammalian target of rapamycin (mTOR) pathway during recovery from an endurance bout after consumption of protein with CHO is similarly limited (16,26).

Few studies have examined the influence of consumption of a whole-food protein–CHO source (i.e., fluid milk) after endurance exercise on indices of subsequent endurance

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Submitted for publication December 2010.
Accepted for publication August 2011.

0195-9131/12/4404-0682/0
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DOI: 10.1249/MSS.0b013e3182364162

performance (18,28,35). However, several studies have clearly demonstrated that protein consumption in combination with CHO enhances initial (31,32,36) or subsequent (2,35) endurance exercise performance. Early postexercise protein–CHO consumption is consistent with previous research investigating pertinent variables such as muscle glycogen, performance, and indices of protein turnover (14,16,26,40). These data highlight the potential for postexercise protein–CHO consumption because adequate glycogen stores affect subsequent exercise performance as well as favorably influence postexercise whole-body protein turnover (WBPTO) (13). Because fluid bovine milk is considered a high-quality protein in that it provides all essential amino acids and the CHO content provided in chocolate milk exceeds that of white milk, fat-free chocolate milk (MILK) holds particular intrigue as a recovery beverage for endurance athletes. Currently lacking are comprehensive studies that effectively examine the influence of post–endurance exercise protein–CHO consumption in a whole-food matrix on measures of protein turnover, glycogen repletion, and exercise performance during the recovery period.

The purpose of the present study was to determine the effect of consuming MILK after a 45-min endurance exercise bout on comprehensive measures of protein-related metabolic responses during exercise recovery. We hypothesized that compared with a CHO-only control beverage (CON), consumption of MILK after exercise would enhance skeletal muscle protein synthesis and associated Akt–mTOR intracellular signaling, attenuate skeletal muscle and whole-body proteolysis, maintain muscle glycogen content, and improve subsequent endurance time to exhaustion (TTE).

METHODS

Subjects

After approval of the studies by the Institutional Review Board for Human Subjects at the University of Connecticut, two similarly matched groups of recreational and club-level male runners volunteered to participate as subjects in distinct research studies. All subjects provided voluntary written informed consent. Individuals were excluded from the studies if responses to a health questionnaire indicated a history of metabolic or cardiovascular abnormalities, gastrointestinal disorders (i.e., lactose intolerance), and use of prescription medication, anabolic steroids, or nutritional/sports supplements besides vitamins and minerals. All subjects refrained from the use of alcohol, nicotine, and caffeine for the duration of the study.

Experimental Design

For studies 1 and 2, participants performed two experimental protocols in random order, separated by 7 d. Each protocol consisted of a 45-min controlled exercise bout followed by a 3-h recovery period during which subjects consumed one of two experimental beverages. Both protocols were identical with the exception of the composition of the beverage consumed during recovery. Subjects randomly consumed a single bolus (480 mL) of either MILK (Nesquik® Ready-to-Drink fat-free chocolate milk; Société des Produits Nestlé S.A., Vevey, Switzerland) or a CON containing 74.0 g of sweetened grape-flavored drink mix (Big Y® Foods, Inc., Springfield, MA) prepared in bottled water. Beverages were isocaloric at 296 kcal. MILK contained 16 g of protein (64 kcal), whereas CON was nonnitrogenous. The CHO content of CON (74 g, 296 kcal) was greater than that of MILK (58 g, 232 kcal). Neither beverage contained fat.

Preliminary Testing

Peak oxygen uptake. Each subject performed a graded exercise test to exhaustion (treadmill running) to determine $\dot{V}O_{2peak}$ via open-circuit spirometry on a metabolic cart (MedGraphics, St. Paul, MN). A constant running speed was maintained while treadmill incline (starting at 0%) increased by 2% every 2 min. $\dot{V}O_{2peak}$ was defined as the oxygen consumption at which the subject's RER was >1.15, HR was ±5 beats·min^{-1} of the age-predicted maximum, or the subject was unable to continue running. Running speed for exercise economy at 65% of $\dot{V}O_{2peak}$ for the 45-min experimental trial runs was determined after a brief recovery from the $\dot{V}O_{2peak}$ test.

Diet and physical activity control. Each subject completed a 3-d food log at baseline that was analyzed for energy intake sufficiency (Nutritionist Pro v. 4.1.0 dietary analysis software; Axxya Systems, Stafford, TX) and to provide an estimate of energy intake needed for weight maintenance throughout study periods. Total daily caloric requirements were calculated from resting energy expenditure data and average daily exercise-induced thermogenesis from a 7-d activity log. Resting energy expenditure was determined using open-circuit spirometry on a metabolic cart (MedGraphics) and exercise-induced thermogenesis estimated by MET associated with a specific activity as detailed by Ainsworth et al. (1). Subjects were provided with copies of their 7-d activity logs and instructed to repeat the same type, duration, and intensity of exercise during the 14 d of the study.

After completing baseline testing, subjects consumed a eucaloric diet throughout the 14 d of the study. Protein intake was prescribed at 1.5 g·kg^{-1}·d^{-1}, fat intake was ≤30% of total daily energy intake, and CHO intake was 6 g·kg^{-1}·d^{-1}. Every meal was prepared by research personnel, in conjunction with the University of Connecticut Dining Services, with subjects' intake monitored and documented during meal times. Subjects were instructed not to consume anything other than water outside of the prepared meals and snacks. Food intake analysis (Nutritionist Pro v. 4.1.0; Axxya Systems) demonstrated no difference in total energy or macronutrient content between trials (data not shown).

Anthropometry. Height was determined using a stationary stadiometer (Health O Meter, Alsip, IL). Body weight was

measured after full void and before breakfast, using a portable digital scale (349KLX; Health O Meter). Percent body fat was determined using scanning dual-energy x-ray absorptiometry (DPX-MD densitometer; Lunar Corp., Madison, WI).

Experimental Protocol Details—Study 1

In study 1, eight individuals participated; however, data for only six participants are included in the final report for muscle protein FSR because of analytical errors. Subjects (n = 8, 23.7 ± 1.6 yr, 1.78 ± 0.03 m, 76.0 ± 3.8 kg, body mass index = 24.0 ± 0.9 kg·m^{-2}, body fat = 14.4% ± 2.0%, $\dot{V}O_{2peak}$ = 53.1 ± 1.6 mL·kg^{-1}·min^{-1}) reported to the Metabolic Assessment Laboratory to begin the experimental protocol on study day 7 (Fig. 1). Each subject completed a 12-h overnight fast after a prepared meal and beverage after the eucaloric diet and had refrained from any resistance exercise for 72 h and aerobic exercise for 24 h before the trial. Upon arrival, the subject was catheterized in an antecubital vein for collection of a baseline blood sample. A primed continuous infusion (2 μmol·kg^{-1}, 0.05 μmol·kg^{-1}·min^{-1}) of L-[*ring*-$^{2}H_5$]phenylalanine (certified sterile and pyrogen-free by Cambridge Isotope Laboratories, Andover, MA) was begun at time 0, and the subject rested, reclined, for 75 min.

The subjects then began the 45-min treadmill exercise bout at 65% of $\dot{V}O_{2peak}$. Indirect calorimetry was used to confirm exercise intensity approximately 5 min into the exercise bout. Treadmill speed was adjusted accordingly if workload was inadequate. Water was provided *ad libitum* during the run. Immediately after the run, a second catheter was inserted into a contralateral hand vein for subsequent blood collection. A percutaneous muscle biopsy was taken under sterile conditions from the lateral portion of the vastus lateralis, and a corresponding blood sample was taken at 0 h of recovery (120 min into the protocol). Immediately after the biopsy, subjects consumed either MILK or CON. Muscle samples were cleaned of visible blood and connective tissue and immediately frozen in liquid nitrogen. Subjects then completed a passive recovery period lasting 3 h, during which second, third, and fourth muscle biopsies were obtained at 0.5 h into recovery (150 min into the protocol), 1 h into recovery (180 min into the protocol), and 3 h or end of recovery (300 min into the protocol). Additional blood samples were collected every 15 min throughout recovery to ensure isotopic steady state and to measure plasma insulin concentrations (DSL-10-1600; Diagnostic Systems Laboratories, Webster, TX). Subjects immediately began the second week of interventions. All treatments were similar to the first week, with the exception of the experimental postexercise beverage, which was the beverage not consumed on the protocol day of the first week.

Determination of FSR. FSR of skeletal muscle protein was calculated using the single pool precursor–product model to determine the rate of tracer incorporation from the muscle free amino acid intracellular pool into bound muscle protein between 0 and 3 h of recovery (120 and 300 min of the protocol) (27). Biopsied muscle samples from the 120- and 300-min protocol time points were used for the Western blotting analysis.

Western blotting. A portion of wet, frozen biopsied muscle (~40 mg) was homogenized (1:9, w/v) in 50 mM of Tris-HCl, 250 mM of mannitol, 50 mM of NaF, 5 mM of sodium pyrophosphate, 1 mM of EDTA, 1 mM of ethyleneglycotetraacetic acid (EGTA), 1% Triton X-100 complete mini protease inhibitor tabs (Roche, Indianapolis, IN), 1 mM of dithiothreitol, 1 mM of benzamidine, 0.1 mM of phenylmethanesulfonyl fluoride, and 5 μg·mL^{-1} of soybean trypsin inhibitor, pH 7.4. Homogenates were then centrifuged at 1500*g* for 3 min at 4°C. The supernatant was then separated and again centrifuged at 3500*g* for 10 min at 4°C. This final supernatant was then assayed for total protein content using Bradford reagents with bovine serum albumin standards (Bio-Rad Laboratories, Hercules, CA). Samples were then combined with appropriate volumes of distilled water and 4× loading buffer, containing 0.3 M of Tris base, 50% glycerol, 8% sodium dodecyl sulfate, 4% 2-mercaptoethanol, and 0.2% bromophenol blue. All samples were stored at −80°C before

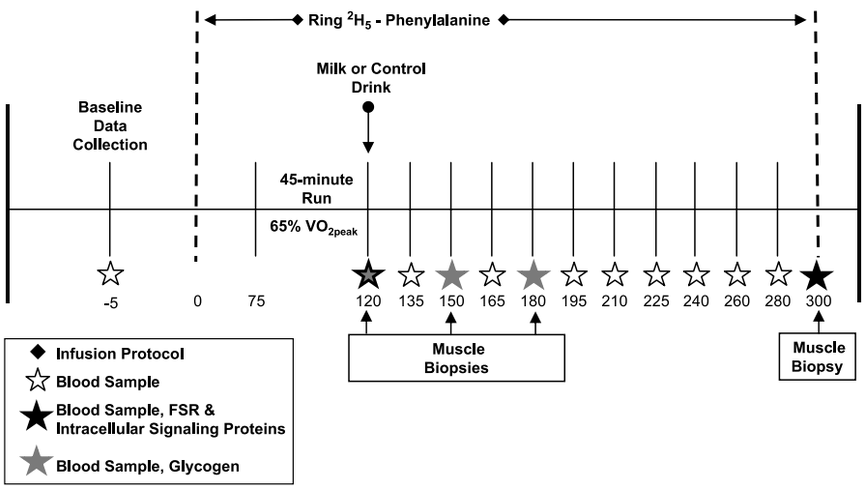


FIGURE 1—Timeline of events of the experimental protocol for study 1 on days 7 and 14. *Numbers* indicate minutes progressed into the protocol.

gel loading. All samples were boiled at 100°C for 3 min before electrophoresis. A total of 30 μg of protein was loaded into each lane of a precast Tris-HCl polyacrylamide gel (Bio-Rad Laboratories). Precision Plus Kaleidoscope protein standards (Bio-Rad Laboratories) were used as molecular weight markers on each gel. Once loaded, gels were electrophoresed in 1× Tris/glycine, 0.1% sodium dodecyl sulfate at 200 V until the bromophenol blue dye reached the bottom of the gel. Proteins were transferred to polyvinylidene fluoride membranes (Bio-Rad Laboratories) at 100 V for 2 h at 4°C using a 20% methanol, 1× Tris/glycine transfer buffer. Once transferred, membranes were briefly air-dried, rewet in 100% methanol, incubated in transfer buffer for 30 s, and placed in a blocking solution for 1 h at room temperature on a tilting rocker. Membranes were blocked in 5% nonfat dry milk (NFDM) in Tris-buffered saline containing 1% Tween-20 (TBST). Once blocked, membranes were placed in their respective primary antibody solutions (described below) and rocked overnight on a tilting platform at 4°C. The next morning, membranes were washed in TBST (one "flash" wash followed by 5 × 5-min soaking washes) and incubated for 1 h in appropriate horseradish peroxidase (HRP)–conjugated secondary antibody solutions (described below) on a rocking platform at room temperature. Membranes were then washed again in TBST (one flash wash and 5 × 5-min soaking washes) and visualized using LumiGLO® chemiluminescent substrate (Cell Signaling Technology, Danvers, MA), Kodak Biomax Light film (Rochester, NY), and Kodak GBX developer/fixer. After visualization, antibodies were stripped from all membranes by placing them in a 2.5% glycine, 2% 2-mercaptoethanol stripping buffer on an orbital shaker at 60°C for 2 h. The membrane containers were placed into sealed plastic bags, and the stripping occurred in a closed-sash fume hood. All membranes were then washed in TBST (3 × 5 min) and blocked in 5% NFDM in TBST. Membranes were again washed (5 × 5 min) and incubated overnight at 4°C on a rocking platform with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primary antibody, used to confirm equal intragel protein loading and to calculate relative phosphorylation. The following morning, membranes were washed, incubated with HRP-conjugated secondary antibody, washed, and visualized as described previously. A scanning densitometry application (ImageJ, from the National Institutes of Health, Bethesda, MD) quantified protein bands (8). Phosphorylation data were expressed relative to total GAPDH by dividing phosphorylation density by the density of the GAPDH protein band and presented as fold change as compared with the 120-min (0 h of recovery) time point. All antibodies were purchased commercially from Cell Signaling Technology. The following are the rabbit-raised primary antibodies (all 1:1000 dilutions in 5% NFDM in 1× TBST) used in these studies: phospho-Akt (Ser^{473}), phospho-eukaryotic initiation factor 4E-binding protein 1 (eIF4E-BP1; $Thr^{37/46}$, γ-subunit), phospho-mTOR (Ser^{2448}), phospho-S6 ribosomal protein (rp-S6; $Ser^{235/236}$), phospho-eukaryotic elongation factor 2 (eEF2; Thr^{56}), phospho-forkhead box protein subgroup O3a (FOXO3a; Ser^{253}), caspase-3, and GAPDH. The goat antirabbit immunoglobulin G HRP-conjugated secondary antibody was diluted 1:2000.

Caspase-3 enzymatic activity assay. The 120- and 300-min protocol time point (0 and 3 h of recovery) muscle biopsy samples were analyzed for caspase-3 activity. The caspase-3 primary antibody used in this study recognizes three distinct forms of the enzyme: the 35-kDa uncleaved, inactive procaspase-3 and the 19- and 17-kDa cleaved, active caspase-3 isoforms. Each individual band was quantitated, as described using ImageJ, with relative densities for the 19- and 17-kDa bands summed. This sum was then divided by the density for the 35-kDa procaspase-3 band, giving relative caspase-3 activation. Each individual band was quantitated, with relative densities for the 19- and 17-kDa bands combined. Relative caspase-3 activity was calculated by summing the relative imaging densities for each band (the inactive procaspase-3 band and two active, cleaved isoforms), then dividing the sum by the density for the procaspase-3 band.

Proteasome enzymatic activity assay. The 120- and 300-min protocol time point (0 and 3 h of recovery) muscle biopsy samples were analyzed for proteasome activity. Mixed muscle tissue aliquots (~40–70 mg), previously stored at −80°C, were homogenized by hand in chilled homogenization buffer (1:7, w/v), containing 20 mM of Tris-HCl, pH 8.2, 0.1 mM of ethylenediaminetetraacetic acid, and 0.04% IGEPAL (Sigma-Aldrich, St. Louis, MO). Immediately before using the buffer, adenosine triphosphate and 2-mercaptoethanol (Bio-Rad Laboratories) were added to 5- and 7-mM final concentrations, respectively. Muscle homogenates were then centrifuged at 13,000*g* for 15 min at 4°C. The protein-containing supernatant was then assayed for total protein content using Bradford reagents with bovine serum albumin standards (Bio-Rad Laboratories) and stored at −80°C for later analysis.

Activity assay samples were run on a 96-well plate format. Samples were prepared in duplicate: one with proteasome inhibitor (100 μg of total protein from the homogenate in 50-mM HEPES/5-mM EGTA buffer also containing the irreversible proteasome inhibitor lactacystin, reconstituted in dimethyl sulfoxide to 50 μM, and a corresponding duplicate containing just 100 μg of protein in HEPES/EGTA (without proteasome inhibitor). All samples were then incubated for 30 min at 37°C. The fluorophore *N*-Succinyl-Leu-Leu-Val-Tyr-7-Amido-4-Methylcoumarin (LLVY-mca; Sigma-Aldrich), reconstituted to 200 μM in dimethyl sulfoxide, was next added to match the volume of the sample in each tube, and all samples were again incubated at 37°C for 60 min. The contents of each tube were loaded in equal volumes to separate wells of a black clear-bottom polystyrene 96-well plate (Corning, Inc., Corning, NY). Fluorescence was read (Molecular Devices SpectraMax Gemini; Sunnyvale, CA), using 380- and 460-nm excitation and emission wavelengths, respectively.

Muscle glycogen determination. Mixed muscle tissue portions (~40–70 mg) from biopsy protocol time points

120, 150, and 180 min (0, 0.5, and 1 h into recovery), previously stored at −80°C, were used for the determination. A phenol–sulfuric acid colorimetric assay described by Lo et al. (20) was used to determine wet muscle glycogen content.

Experimental Protocol Details—Study 2

Subjects (n = 6, 21.3 ± 1.2 yr, 1.75 ± 0.02 m, 71.3 ± 2.7 kg, body mass index = 23.2 ± 0.7 kg·m^{-2}, body fat = 12.5% ± 1.0%, $\dot{V}O_{2peak}$ = 54.0 ± 0.8 mL·kg^{-1}·min^{-1}) reported to the Metabolic Assessment Laboratory to begin the experimental protocol on study day 7 (Fig. 2). Preprotocol fasting conditions and exercise restriction and the protocol exercise, beverage consumption, and recovery conditions were similar to those of study 1. An experimental WBPTO protocol detailed by Gaine et al. (7) was used, using a bolus injection of ^{13}C-bicarbonate (0.3 mg·kg^{-1}) to prime the bicarbonate pool and a primed continuous infusion (4 μmol·kg^{-1}, 4.8 μmol·kg^{-1}·h^{-1}) of L-[1-^{13}C]leucine (certified sterile and pyrogen-free by Cambridge Isotope Laboratories). Baseline and all subsequent blood and breath samples were collected for analysis of plasma ^{13}C-α-ketoisocaproate (KIC) enrichment and $^{13}CO_2$ enrichment determination by gas chromatography and isotope ratio mass spectroscopy, respectively, by a commercial laboratory (Metabolic Solutions, Nashua, NH).

Plasma ^{13}C-α-KIC and breath $^{13}CO_2$ enrichment at four time points during recovery (225, 240, 255, and 270 min into the protocol) were evaluated to confirm isotopic steady-state conditions. Steady-state was assumed when the coefficient of variation of the percent enrichment and atom percent excess (APE) values at isotopic plateau was <10%. Data from the four time points were averaged for each subject, and group means were determined. WBPTO variables leucine rate of appearance (Ra; an indicator of protein breakdown), leucine oxidation (Ox), and nonoxidative leucine disposal (NOLD; an indicator of protein synthesis) were calculated using the reciprocal pool model (11). Net leucine balance (NET) was calculated by subtracting leucine Ra from NOLD.

TTE test. Immediately after the final blood and breath sample collection, catheters were removed, and the subject was moved to the treadmill for a performance test. Each subject completed a pretest warm-up by walking at 3 mph at 0% incline for 4 min. Treadmill speed was then increased to the speed set during the $\dot{V}O_{2peak}$ determination during preliminary testing, and the subject ran an additional 4 min at 0% incline. Treadmill incline was then immediately increased to that achieved at $\dot{V}O_{2peak}$ test cessation for each subject. Subjects were instructed to run until volitional exhaustion. No timepieces were available to the subjects during the test. TTE was calculated as the time elapsed from the time of treadmill elevation to the time of volitional exhaustion.

Statistics. Baseline subject characteristics are described using descriptive statistics. Repeated-measures ANOVA was used to determine the effects of time and beverage type on protein phosphorylation, activity of caspase-3 and the 26S proteasome, plasma insulin, WBPTO breath $^{13}CO_2$ APE, and muscle glycogen content during recovery. In the event of ANOVA significance, Bonferroni *post hoc* adjustment was used to reduce type I error rate when making pairwise comparisons. Paired t-tests were used to evaluate differences between the MILK and CON trials for FSR; WBPTO variables Ox, Ra, NOLD, and NET; and TTE. An α level set *a priori* at $P \leq 0.05$ was considered significant. All data were analyzed using SPSS for Windows 11.0.1 (SPSS, Inc., Chicago, IL).

RESULTS

Study 1

Muscle FSR. Mixed muscle protein FSR in MILK (0.11%·h^{-1} ± 0.01%·h^{-1}) was 38% higher than that in CON (0.08%·h^{-1} ± 0.01%·h^{-1}) during the 3-h recovery

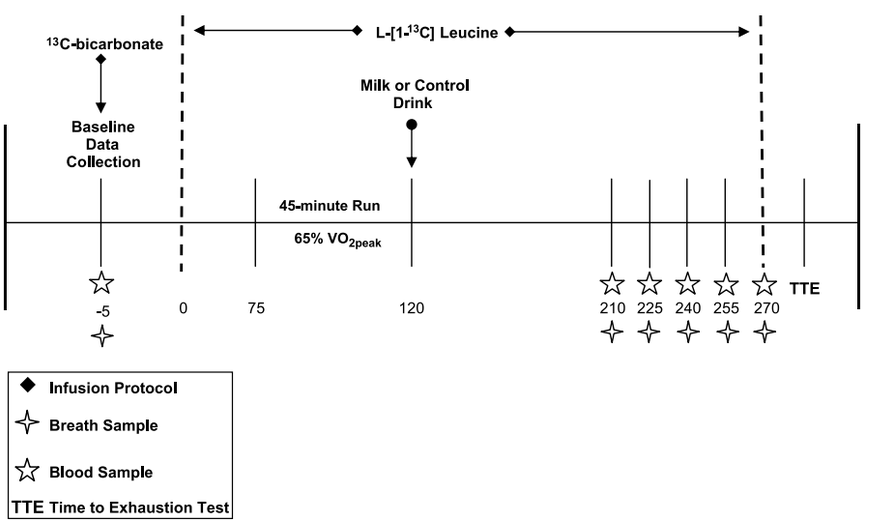


FIGURE 2—Timeline of events of the experimental protocol for study 2 on days 7 and 14. *Numbers* indicate minutes progressed into the protocol.

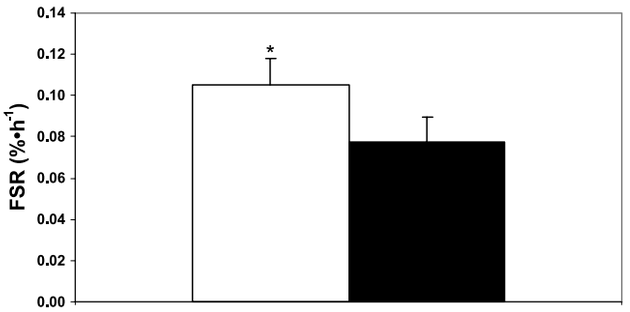


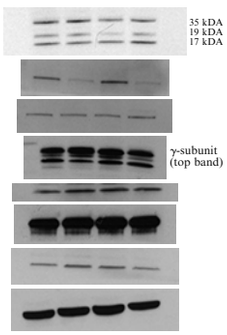
FIGURE 3—Mixed muscle protein FSR during 3 h of recovery from 45 min of running at 65% of $\dot{V}O_{2peak}$ after consuming either MILK (*open bar*) or an isocaloric CON (*solid bar*). Data are means ± SE, $n = 6$. *Difference from CON ($P = 0.05$).

period ($P = 0.05$; Fig. 3). Enrichment of plasma L-[*ring*-$^2H_5$] phenylalanine indicates isotopic steady state during the recovery period (Fig. 4A).

Signaling protein phosphorylation. A 1.15-fold change in eIF4E-BP1 phosphorylation ($Thr^{37/46}$) in the MILK trial during 3 h of recovery was observed (Table 1). Suppressed phosphorylation of Akt (Ser^{473}) occurred in the MILK (−3.10-fold change) and CON (−2.74-fold change) trials. FOXO3a phosphorylation (Ser^{253}) was attenuated at the end of recovery in the CON trial (−1.51-fold change), whereas no effect was observed in MILK. There was no change in phosphorylation of mTOR, rp-S6, or eEF2 in either the MILK or the CON trial during recovery.

TABLE 1. Effect of MILK versus CON consumption after endurance exercise on phosphorylation of translational signaling proteins and proteolytic protein activity during 3 h of recovery.

			MILK		CON	
	MILK	CON	0 h	3 h	0 h	3 h
26S proteasome	1.05 ± 0.06	1.39 ± 0.18*				
Caspase-3	−2.60 ± 0.45*	1.10 ± 0.13				
Akt	−3.10 ± 0.79*	−2.74 ± 0.71*				
mTOR	1.50 ± 0.39	1.34 ± 0.20				
eIF4E-BP1	1.15 ± 0.10*	1.02 ± 0.09				
rp-S6	1.55 ± 0.46	1.90 ± 0.76				
eEF2	1.02 ± 0.09	1.05 ± 0.05				
FOXO3a	1.11 ± 0.06	−1.51 ± 0.15*				
GAPDH	—	—				



Signaling protein and proteolytic activity data are relative to GAPDH expression and presented as fold change relative to 0 h of recovery. Data are means ± SE.
* Significant change from 0 h ($P < 0.05$).

Proteolytic component activity. Caspase-3 activity was lower at the end of 3 h of recovery (300 min into protocol) in the MILK trial (−2.60-fold change) (Table 1), whereas no change was observed for CON. Activity of the 26S proteasome was greater at the end of recovery for CON (1.39-fold change); however, there was no change in proteasome activity during recovery in the MILK trial.

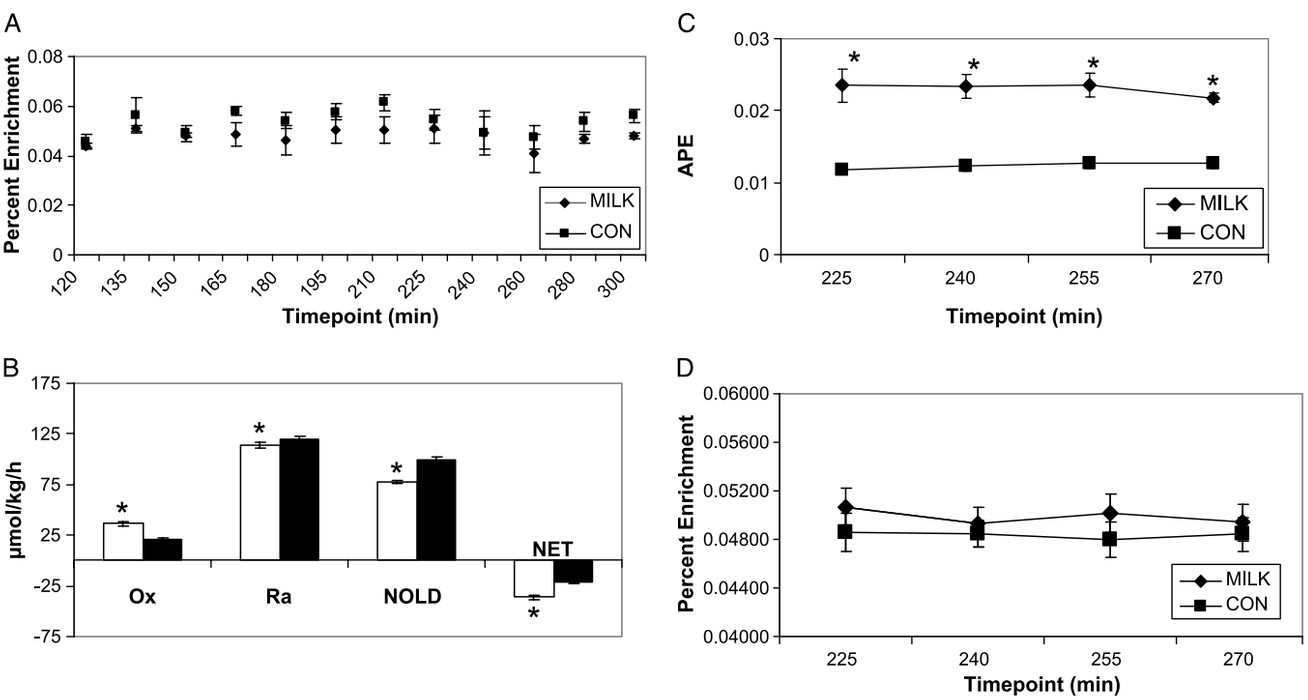


FIGURE 4—A, Plasma percent enrichment of L-[*ring*-$^2H_5$]phenylalanine. Data are means ± SE, $n = 8$. B, WBPTO at the beginning and end of 3 h of recovery from 45 min of running at 65% of $\dot{V}O_{2peak}$ after consuming either MILK (*open bars*) or an isocaloric CON (*solid bars*). C, APE of breath $^{13}CO_2$. D, Percent enrichment of plasma ^{13}C-α-KIC during recovery, demonstrating isotopic steady state. Data for B, C, and D are means ± SE, $n = 6$. *Difference from CON ($P \leq 0.05$).

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Plasma insulin. Baseline (protocol time 0) insulin concentrations were similar at approximately 6 μIU·mL^{-1} between the MILK and the CON trials (Fig. 5). Insulin levels tended to be higher in the MILK trial (78.4 ± 14.2 μIU·mL^{-1}) at 0.5 h into recovery (0.5 h after beverage consumption, also 150 min into protocol) than in CON (42.8 ± 5.1 μIU·mL^{-1}) (P = 0.08). Insulin concentration returned to baseline values in both trials by 3 h into recovery (300 min into protocol).

Muscle glycogen content. Glycogen content was not different between MILK and CON trials at 0 h of recovery (120 min into protocol; 6.4 ± 1.3 and 5.7 ± 0.6 g of glycogen per 100 g of tissue, respectively), at 0.5 h of recovery (0.5 h after beverage consumption, also 150 min into protocol; 6.5 ± 0.9 vs 5.4 ± 0.5 g of glycogen per 100 g of tissue), or at 1 h of recovery (1 h after beverage consumption, also 180 min into protocol; 6.4 ± 0.9 vs 5.4 ± 0.6 g of glycogen per 100 g of tissue).

Study 2

WBPTO. Leucine Ra and NOLD during recovery were 5% (P = 0.05) and 22% (P < 0.01) lower, respectively, for MILK relative to CON (Fig. 4B). However, leucine Ox was 77% greater during recovery in the MILK trial compared with CON (P < 0.01). Therefore, NET was 77% more negative during recovery from endurance exercise for MILK than for CON (P < 0.01). Enrichment of breath $^{13}CO_2$ (Fig. 4C) and plasma ^{13}C-α-KIC (Fig. 4D) during recovery demonstrates steady-state kinetics.

TTE. Treadmill TTE for MILK (250 ± 43 s) was 23% greater than that for CON (203 ± 31 s) (P = 0.03).

DISCUSSION

The major findings in this study were elevated kinetic measures of skeletal muscle protein synthesis and suppressed whole-body protein breakdown during recovery from endurance exercise after consuming MILK compared with a protein-free CHO-only beverage. Also notable was the concurrent heightened phosphorylation state of eIF4EBP-1, a protein in the Akt–mTOR signaling pathway and attenuated proteolytic activity. These data are unique in that they reflect skeletal muscle and WBPTO after whole-food consumption during recovery from endurance exercise. In addition, these studies were conducted in the context of a controlled diet and consistent routine exercise training.

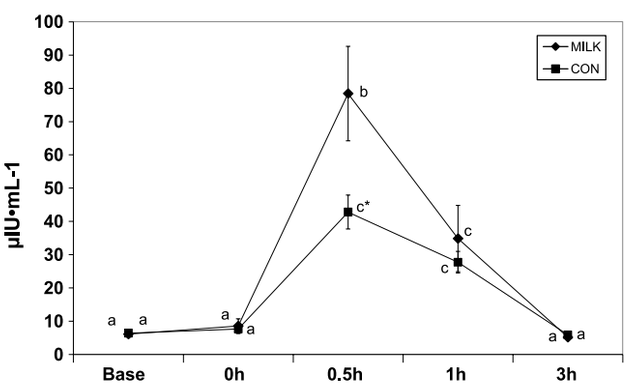

FIGURE 5—Plasma insulin concentration during 3 h of recovery from 45 min of running at 65% of $\dot{V}O_{2peak}$ after consuming either MILK or an isocaloric CON. Data are means ± SE, n = 8. Comparisons sharing different *letters* are statistically significant. *Trend toward significant difference from CON at 0.5 h after exercise (P = 0.08). Base, baseline sample at beginning of protocol; 0 h, 0 h after exercise; 0.5 h, 0.5 h after exercise; 1 h, 1 h after exercise; 3 h, 3 h after exercise.

The greater FSR during recovery observed in study 1 is consistent with previously observed increases in muscle protein FSR after endurance exercise or a combination of exercise and protein-containing recovery nutrition (12,22,39). A practical aspect of this investigation, in the context of stable isotope kinetics determinations, is the demonstrated increase in protein synthesis after the ingestion of a single bolus of the milk beverage immediately after the exercise bout. Researchers in previous studies administered the feedings to their subjects as lesser volume repeated boluses throughout recovery (12,25). We demonstrated that a similar volume of milk can be consumed as a single bolus and elicit enhanced muscle protein synthesis. Although single-bolus ingestion is not new, the present data demonstrate a practical alternative for supporting skeletal muscle protein synthesis during recovery using whole food and endurance exercise, neither of which has been adequately explored in the literature.

An important consideration for individuals engaging in regular endurance exercise is proper nutrition to minimize breakdown and promote synthesis of whole-body protein. Habitual protein intake of 1.5 g·kg^{-1}·d^{-1}, the amount prescribed in the present study, was determined by Gaine et al. (7) to be more effective in decreasing protein breakdown in endurance athletes during recovery from exercise than the recommended daily allowance of 0.8 g·kg^{-1}·d^{-1}. Moreover, provision of milk after exercise in the present study yielded data corroborating findings by Miller et al. (24), who also observed attenuated Ra (breakdown) and elevated Ox during recovery from an endurance run during which fluid skim milk was consumed. One might speculate that the suppression in NOLD (synthesis) observed by Miller et al. (25) and the present authors is indicative of reduced proteolysis after the exogenous provision of protein via milk consumption.

The present observation of increased phosphorylation of eIF4E-BP1 during recovery in the MILK trial was expected, on the basis of postexercise feeding studies in resistance exercise (6) and rodent endurance models (26). However, an unexpected outcome was the reduction in Akt phosphorylation (Ser^{473}) in both trials. Enhanced phosphorylation of this protein has been observed during recovery in previous studies (16,22). However, there is scientific precedence showing reductions in Akt phosphorylation during recovery from an endurance exercise bout. Wilkinson et al. (39) showed that Akt phosphorylation markedly decreased immediately after exercise through a 4-h recovery interval after a 45-min exercise bout similar to that in the present study. Training effect is a viable explanation; Wilkinson et al. (39) observed *increased* Akt phosphorylation after

a similar bout in untrained subjects. However, the likely explanation for the observed reduction in Akt phosphorylation was the timing of the muscle biopsy sampling. No sample was taken earlier than 1 h after exercise cessation. The early time course of Akt (Ser^{473}) phosphorylation peaking 30–60 min after endurance exercise has been documented (3). In addition, although enhanced phosphorylation of eIF4E-BP1 in the MILK trial during recovery supports the greater FSR observation, it would be unreasonable to make an absolute assumption of association. Enhanced translational signaling is not necessarily a definitive indicator of increased protein synthesis rates (9). This finding remains intriguing nonetheless.

The proteolytic activity influenced by MILK consumption was notable and may be explained by the observed insulin response. Activity of caspase-3, the cysteine protease responsible for cleavage of the actomyosin protein in skeletal muscle, was reduced in the MILK group. In addition, consuming MILK maintained both the phosphorylation state of FOXO3a, an atrogene transcription factor, and the activity of the 26S proteasome, the machinery of cellular protein degradation. These specific data are consistent with previously noted effects of insulin signaling through the insulin-like growth factor 1/phosphatidylinostisol 3 kinase (PI3K)/Akt pathway. In the absence of insulin signaling, FOXO3a is dephosphorylated because PI3K is deactivated, allowing subsequent atrogene transcription (17). Similarly, PI3K inactivation promotes caspase-3–induced actin fragmentation from intact skeletal muscle protein (17). Insulin signaling, then, may have rescued a catabolic condition in the MILK group by diminishing proteolysis.

The insulin response to each beverage in the present study may support the proteolysis outcomes. Although not significant, the 83% higher plasma insulin concentration after consuming MILK compared with the CHO-only beverage at the 150-min time point (30 min after beverage consumption and 30 min into the recovery interval) was physiologically relevant. These data are consistent with findings by Williams et al. (40), who found a 92% greater insulin response during recovery in subjects consuming a protein–CHO beverage immediately after a 2-h cycling bout compared with a CHO-only beverage. Greater insulin signaling at PI3K after MILK consumption, then, may have been an avenue of the observed inhibition of actomyosin cleavage by caspase-3, maintained phosphorylation of FOXO3a, and subsequent suppression of the 26S proteasome. All of these events may have contributed to the decreased whole-body protein breakdown observed. The reduction in Akt phosphorylation observed does not support such a mechanism, however. Post–endurance exercise data by Louis et al. (21) and Harber et al. (10) demonstrating messenger RNA induction of FOXO3a suggest that nuclear translocation due to deprotection (dephosphorylation) from Akt occurred. Again, caution is advised when assuming that molecular activity is indicative of kinetic outcomes (9).

One explanation for the differences noted in protein turnover outcomes between the beverage trials in the present study is that the provision of amino acids in the MILK consumed contributed to the amino acid pool and thereby inhibited proteolysis. Smith et al. (33) reported postprandial increases in plasma amino acid concentration after consumption of fluid milk. Indeed, feeding amino acids during endurance exercise has been shown to attenuate phenylalanine release from skeletal muscle in humans (23). A similar response to milk ingestion in the present study is viable. Further, Capel et al. (4) demonstrated suppression of ubiquitin–proteasome pathway–dependent skeletal muscle proteolysis in rats by amino acid feeding, similar to the outcomes in the present study.

The exercise bout implemented in the present study was not sufficient to induce glycogen depletion. This limitation is acknowledged and is consequent to the constraints of the experimental protocol specific to assessment of skeletal muscle protein synthesis supporting glycogen resynthesis (18). That postexercise muscle glycogen content was maintained in the present study is consistent with previous research (16,37) and, therefore, supports our hypothesis. Although the addition of protein to 50–75 g of CHO (i.e., MILK) supported muscle glycogen maintenance as effectively as CHO consumption only, the protein–CHO composition of MILK may have imparted benefits to subsequent performance over and above CON, independent of the contribution from glycogen, and in support of skeletal muscle and whole-body protein utilization.

Studies have shown that consuming protein–CHO nutrition versus CHO only, matched for CHO, improves TTE by 13%–40% during prolonged endurance exercise (30,31). Combined protein–CHO nutrition is no more beneficial than CHO only, however, when matched for calories and when glycogen stores are already impaired (29,36). Yet, during brief, late-stage, extremely intense portions of a performance test, similar in intensity to that in the present study, enhanced performance has been observed relative to CHO-only supplementation (32). There is no clear mechanism by which enhanced TTE observed in the present study after MILK consumption can be explained. We recognize that failure to blind the beverages and the controversy surrounding the use of TTE as an appropriate performance test (15) may have influenced performance outcome independent of substrate availability and utilization. As performance was a point of interest secondary to protein turnover measures in the present study, we advocate that more tightly controlled studies be conducted to investigate the benefits of fluid milk on subsequent exercise performance.

In summary, relative to a CHO-only beverage, consumption of MILK after an endurance exercise bout significantly increased skeletal muscle protein synthesis, attenuated whole-body proteolysis, enhanced phosphorylation of eIF4E-BP1, and suppressed or maintained molecular activity of protein breakdown during recovery. In addition, milk consumption was as effective as the control at maintaining muscle glycogen during the recovery period, and performance in a subsequent exercise bout was enhanced compared with the control. These outcomes were consistent with research

hypotheses. Although further research is needed to elucidate mechanisms of performance enhancement, the data suggest that endurance athletes or individuals who exercise aerobically can consider MILK as a viable option for postexercise nutrition to support skeletal muscle and whole-body protein recovery.

This study was supported by a grant from Dairy Management, Inc.

The authors thank Cosby Jolley, Dr. Robert R. Wolfe, and Dr. Arny A. Ferrando for their technical guidance during the sample preparation for the isotopic mass spectrometry analysis and the study volunteers for their dedication and time.

The authors of the present study declare no conflicts of interest.

Results from the present study do not constitute endorsement by the American College of Sports Medicine.

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