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RESEARCH ARTICLE

Coingestion of protein and carbohydrate in the early recovery phase, compared with carbohydrate only, improves endurance performance despite similar glycogen degradation and AMPK phosphorylation

Marius A. Dahl,[1]* José Lisandro Areta,[1,2]* Per Bendix Jeppesen,[3] Jesper Bratz Birk,[4] Egil I. Johansen,[1] Thorsten Ingemann-Hansen,[5] Mette Hansen,[5] Bjørn Steen Skålhegg,[6] John L. Ivy,[7] Jørgen F. P. Wojtaszewski,[4] Kristian Overgaard,[5] and Jørgen Jensen[1,4]

[1]*Department of Physical Performance, Norwegian School of Sports Sciences, Oslo, Norway;* [2]*Research Institute for Sport and Exercise Sciences, Liverpool John Moores University, Liverpool, United Kingdom;* [3]*Department of Clinical Medicine, Aarhus University, Aarhus, Denmark;* [4]*Section of Molecular Physiology, Department of Nutrition, Exercise and Sports, Faculty of Science, University of Copenhagen, Copenhagen, Denmark;* [5]*Department of Public Health, Aarhus University, Aarhus C, Denmark;* [6]*Department of Nutrition, Division for Molecular Nutrition, University of Oslo, Oslo, Norway; and* [7]*Department of Kinesiology and Health Education, University of Texas at Austin, Austin, Texas*

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Dahl MA, Areta JL, Jeppesen PB, Birk JB, Johansen EI, Ingemann-Hansen T, Hansen M, Skålhegg BS, Ivy JL, Wojtaszewski JF, Overgaard K, Jensen J. Coingestion of protein and carbohydrate in the early recovery phase, compared with carbohydrate only, improves endurance performance despite similar glycogen degradation and AMPK phosphorylation. *J Appl Physiol* 129: 297–310, 2020. First published June 25, 2020; doi:10.1152/japplphysiol.00817.2019.—The present study compared the effects of postexercise carbohydrate plus protein (CHO+PROT) and carbohydrate (CHO)-only supplementation on muscle glycogen metabolism, anabolic cell signaling, and subsequent exercise performance. Nine endurance-trained males cycled twice to exhaustion (muscle glycogen decreased from ~495 to ~125 mmol/kg dry wt) and received either CHO only (1.2 $g \cdot kg^{-1} \cdot h^{-1}$) or CHO+PROT (0.8/0.4 $g \cdot kg^{-1} \cdot h^{-1}$) during the first 90 min of recovery. Glycogen content was similar before the performance test after 5 h of recovery. Glycogen synthase (GS) fractional activity increased after exhaustive exercise and remained activated 5 h after, despite substantial glycogen synthesis (176.1 ± 19.1 and 204.6 ± 27.0 mmol/kg dry wt in CHO and CHO+PROT, respectively; $P = 0.15$). Phosphorylation of GS at *site 3* and *site 2+2a* remained low during recovery. After the 5-h recovery, cycling time to exhaustion was improved by CHO+PROT supplementation compared with CHO supplementation (54.6 ± 11.0 vs. 46.1 ± 9.8 min; $P = 0.009$). After the performance test, muscle glycogen was equally reduced in CHO+PROT and CHO. Akt Ser^{473} and p70s6k Thr^{389} phosphorylation was elevated after 5 h of recovery. There were no differences in Akt Ser^{473}, p70s6k Thr^{389}, or TSC2 Thr^{1462} phosphorylation between treatments. Nitrogen balance was positive in CHO+PROT (19.6 ± 7.6 mg nitrogen/kg; $P = 0.04$) and higher than CHO (−10.7 ± 6.3 mg nitrogen/kg; $P = 0.009$). CHO+PROT supplementation during exercise recovery improved subsequent endurance performance relative to consuming CHO only. This improved performance after CHO+PROT supplementation could not be accounted for by differences in glycogen metabolism or anabolic cell signaling, but may have been related to differences in nitrogen balance.

NEW & NOTEWORTHY Endurance athletes competing consecutive days need optimal dietary intake during the recovery period. We report that coingestion of protein and carbohydrate soon after exhaustive exercise, compared with carbohydrate only, resulted in better performance the following day. The better performance after coingestion of protein and carbohydrate was not associated with a higher rate of glycogen synthesis or activation of anabolic signaling compared with carbohydrate only. Importantly, nitrogen balance was positive after coingestion of protein and carbohydrate, which was not the case after intake of carbohydrate only, suggesting that protein synthesis contributes to the better performance the following day.

Akt/PKB; exercise; glycogen synthase; nitrogen balance; protein synthesis

INTRODUCTION

Many studies have shown that coingestion of protein and carbohydrate after exhaustive endurance exercise improves recovery of performance more than carbohydrate only (12, 54, 58, 60, 66), but the reason for improved performance remains unknown. Carbohydrate is the major energy substrate during prolonged high-intensity endurance exercise (1, 16, 48, 54, 63), and fatigue develops when the glycogen stores becomes low (6, 16). Although proteins are not considered a major energy substrate during exercise, metabolism of leucine increases during exercise (44, 69), and oxidation of branched-chain amino acids in skeletal muscle is required for satisfactory endurance capacity (59). Moreover, utilization of amino acids increases during exercise when glycogen content is low (22, 36). Endurance athletes have a high-protein requirement, and as much as 1.5–2.0 g/kg body wt seems necessary to avoid negative nitrogen balance (29, 54, 60, 61). Therefore, many elite cyclists ingest protein after training to stimulate the recovery process (14, 57).

Coingestion of protein and carbohydrate has been reported to increase the rate of glycogen synthesis compared with carbohydrate only (5, 70). The mechanisms contributing to recovery of performance after endurance exercise include glycogen synthesis (23). However, protein intake after exercise stimulates protein synthesis (15, 21, 37, 65), limits muscle

* M. A. Dahl and J. L. Areta are to be considered first authors.
Correspondence: J. Jensen (e-mail: jorgen.jensen@nih.no).

damage (9), modulates transcription (51), and increases activation of anabolic signaling pathways (12, 49). Some studies have not found that coingestion of protein and carbohydrate recovers performance better than carbohydrate only (5, 43, 47, 52, 53). The reasons that protein intake in the recovery period did not improve performance in these studies may be due to the type and duration of exercise before the dietary interventions, the length of recovery period, nitrogen balance, and the test used to evaluate performance. Importantly, it is necessary to develop protocols where coingestion of protein and carbohydrate consistently improves recovery of performance better than intake of carbohydrate only. Without such protocols, it is impossible to illuminate the mechanisms responsible for the improved performance after intake of protein.

Muscle biopsies have been taken in only one study where coingestion of protein and carbohydrate improved recovery of endurance performance relative to carbohydrate alone (12). Ferguson-Stegall et al. (12) showed that glycogen synthesis was similar, but activation of anabolic signaling was increased with coingestion of protein and carbohydrate. Many other studies have reported improved activation of anabolic signaling after intake of protein (42, 46, 49, 51, 56, 65), but none of these studies investigated the effect of protein intake on performance.

In two recent studies, we observed improved exercise performance after coingestion of protein and carbohydrate when provided during the first 90 min of recovery from exhaustive exercise compared with intake of carbohydrate only (54, 60). However, the molecular mechanisms responsible for this improved performance were not investigated in these studies. Therefore, our goal was to use the same exercise and dietary protocol used successfully in our previous studies (54, 60) in combination with muscle biopsies to investigate the possible mechanisms contributing to the improved exercise performance associated with postexercise supplementation of protein plus carbohydrate (CHO+PROT). The first aim of the present study was to compare the effects of a CHO+PROT supplement to an isocaloric CHO supplement ingested during the first 2 h of a 5-h recovery period on muscle glycogen metabolism and activation of anabolic cell signaling. The second aim was to investigate glycogen use and the anabolic cell signaling response to an exercise performance test to exhaustion after 5 h of recovery, when performance was enhanced by a CHO+PROT supplement relative to an isocaloric CHO supplement.

MATERIALS AND METHODS

Nine males training for competition in a triathlon ($n = 2$) or cycling (mountain biking; $n = 7$) completed the study. Inclusion criteria were *1*) bicycle training more than twice a week for the last 6 mo, *2*) $\dot{V}O_{2max} \geq 50$ ml·kg^{-1}·min^{-1}, *3*) age 18–40 yr, and *4*) no known diseases. Characteristics of the participants were age: 26.7 ± 1.7 yr; weight: 76.4 ± 3.2 kg; height: 182.4 ± 2.2 cm; maximal heart rate: 188.0 ± 2.2 bpm and $\dot{V}O_{2max}$: 58.1 ± 1.7 ml·kg^{-1}·min^{-1}.

The participants were informed individually about the study and biopsy procedures, and each participant provided informed written consent. The study was approved by the Regional Ethical Committee of Midtjylland, Denmark (Journal no. 1-10-72-23-13) and conducted in accordance to the principles from the Declaration of Helsinki. The study was a double-blinded, crossover design, with one week between the two interventions. Random assignment into groups was done by minimization (55) using publicly available software (Minim: Allocation by minimization in clinical trials).

Testing of $\dot{V}O_{2peak}$ and the Incremental Test

Tests were performed on an SRM cycle ergometer (SRM, Jülich, Germany) adjusted individually to the participants specifications. Oxygen uptake and CO_2 production were measured with an AMIS 2001 analyzer (Innovation, Odense, Denmark) (27). The analyzer was calibrated with a gas mixture containing 16.5% O_2 and 4.0% CO_2, according to company instructions. During testing, gas sampling was averaged over 10-s periods. Mean laboratory temperature and humidity during the testing were 21.9 ± 0.2°C and 41.6 ± 1.2%, respectively.

Incremental test. On the first test day, participants performed *1*) an incremental exercise test to establish the relationship between work rate and oxygen uptake, and *2*) measure peak oxygen consumption ($\dot{V}O_{2peak}$). Initially, there was a short (3–5 min) warm-up at 100 W during which participants selected a cadence between 90 and 100 revolutions per min (RPM). This self-selected RPM was used in all testing throughout the study. The incremental test then started at 125–175 W depending on training condition and increased 25 W every 5th min. $\dot{V}O_2$ was measured during the last 90 s of each load. After 4 min of cycling at each exercise work rate, a capillary blood sample (Accu-Check, Safe-T-Pro Plus; Manheim, Deutschland) was taken for measurement of blood lactate and glucose. Capillary blood glucose was measured with a HemoCue Glucose 201+ analyzer (Angelholm, Sweden). For lactate analyses, a microhematocrit tube (55 μL; Radiometer, Copenhagen, Denmark) was filled, and 23 μL of blood was immediately pipetted into a YSI Analyzer (Yellow Springs Instruments 1500 SPORT, Yellow Springs, OH). The incremental test terminated when the blood lactate concentration was higher than 4 mM. The YSI Analyzer was calibrated with a standard of 5 mM lactate each day. Heart rate (HR) was measured continuously during all testing with a Polar RS 800-CX (Kempele, Finland).

Testing of peak oxygen consumption ($\dot{V}O_{2peak}$). After the incremental test, participants were allowed 5–10 min of rest before the $\dot{V}O_{2peak}$ test. The $\dot{V}O_{2peak}$ test started at the last workload with blood lactate below 4 mM during the incremental test. The load was increased by 25 W every 60 s until exhaustion. $\dot{V}O_{2peak}$ was estimated as the highest 1-min average for $\dot{V}O_2$. Linear regression was used to establish the relationship between aerobic workload and $\dot{V}O_2$ during the incremental test, and the workload corresponding to 70% of $\dot{V}O_{2peak}$ was calculated for subsequent testing.

Diet and Training before Baseline Biopsy and Interventions

Participants were instructed to keep a normal diet and refrain from any protein supplementation the last 24 h before the baseline biopsy. Training was allowed the day before, but restricted to easy endurance exercise with the duration no longer than 60 min. Both the diet and training were recorded and repeated before all test days. Participants fasted overnight (the last meal was consumed at 9.00 PM) before test days. If the participants lived a distance further than 2 km away from the laboratory, they were instructed to come by car or public transportation.

Baseline muscle and blood sampling. Participants reported to the laboratory at 8.00 AM after an overnight fast. The muscle biopsy was taken from the vena lateralis. After removing hair with a razor from the thigh area, the skin was disinfected with chlorhexidine (0.5% SAiD). Then 2 mL lidocaine (10 mg/ml) was injected subcutaneously above and beneath the muscle fascia. A small incision (5 mm) was made in the skin and muscle fascia with a scalpel. Any bleeding was stopped by pressure on the wound for ~5 min. Biopsies were taken with a Bergström needle modified for suction. The tissue was quickly examined and frozen in liquid nitrogen (−196°C), and stored at −80°C until further analysis. A venous blood sample (8-mL heparinized tube) was taken from the vena basilica in a supine position. Blood

samples were kept on ice until centrifugation (10 min at 4°C and 1300 *g*). After centrifugation, the plasma was pipetted in Eppendorf tubes and stored at −80°C until further analysis. Lastly, a capillary blood sample was taken for glucose analysis (Hemocue Glucose 201+, Angelholm, Sweden).

Familiarization Trial

A preliminary trial was performed to familiarize the participants with cycling and adjusting workload on the SRM ergometer. The trial started with a standardized warm-up consisting of three sets of 4-min cycling at workloads corresponding to 50, 55, and 60% of $\dot{V}O_{2peak}$. The same warm-up was used in all subsequent testing throughout the study. After a warm-up, the workload was set to the estimated workload corresponding to 70% of $\dot{V}O_{2peak}$. $\dot{V}O_2$ was measured after 4 min over 90 s. If the $\dot{V}O_2$ was more than 1 $ml \cdot kg^{-1} \cdot min^{-1}$ from the calculated 70% of $\dot{V}O_{2peak}$, workload was adjusted accordingly, and $\dot{V}O_2$ was measured 4 min later. The participants were allowed a 15-min break after the workload was adjusted. Afterward, they cycled 30 min at the workload corresponding to 70% of $\dot{V}O_{2peak}$ ($W_{70\%}$) during which time $\dot{V}O_2$, capillary blood samples, and rate of perceived exertion (RPE – Borg scale) were recorded every 10 min. The familiarization trial was completed at least 3 days after the resting biopsy.

Dietary Intervention Days

On the two experimental test days, participants reported to the laboratory at 7:30 AM Initially, resting $\dot{V}O_2$, respiratory exchange ratio (RER), and HR were measured for 10 min, while the participants were in a supine position (results obtained from the last 5 min). After the 10-min rest, venous and capillary blood samples were taken (Fig. 1). The participants were then asked to empty their bladder before the exercise started.

Initial glycogen-depleting exercise. On test days, the initial exercise session began at 8:00 AM, with the aim of depleting muscle glycogen. The exercise started with a standardized warm-up, and after 5 min of rest, the participants started cycling at 70% of $\dot{V}O_{2peak}$. The exercise was divided into cycling sessions separated by 5-min breaks. The first session lasted 30 min, and all subsequent sessions lasted 20 min. The participants were reminded to drink water every 10 min of cycling. $\dot{V}O_2$ and RER were measured over 90 s after 3.5 min of the first session, at the end of each session, and the last 60 s before exhaustion. Following measurement of $\dot{V}O_2$, and before measurement of capillary lactate and glucose, rating of perceived exertion was recorded. HR was measured throughout the performance test. Participants cycled until exhaustion at a workload corresponding to 70% of $\dot{V}O_{2peak}$. Then after a 5-min rest, a series of 1-min sprints at a workload corresponding to 90% of $\dot{V}O_{2peak}$, interspersed with 1-min breaks, were performed until the participants could not maintain their predefined peddling cadence. Capillary glucose and lactate were measured at exhaustion as described above.

Tissue sampling during recovery. After the initial glycogen-depleting exercise, a Venflon catheter (BD Venflon Pro, Helsingborg, Sweden) connected to a three-way valve (BD Connecta, Helsingborg, Sweden) was inserted in an antecubital vein for blood sampling. A total of nine venous blood samples were collected during recovery: 0, 30, 60, 90, 120, 150, 180, 240, and 300 min after exercise completion (Fig. 1). All venous blood samples were taken on lithium-heparinized tubes and treated in the same manner as the resting sample. The catheter was flushed with saline following each blood collection.

Muscle biopsies. After the first blood sample, musculus vastus lateralis was prepared for muscle biopsy, as described for the resting biopsy. The 5-h recovery period started when the biopsy was taken ~15 min after the exhaustive exercise protocol. A second biopsy was taken from the opposite leg after the 5-h recovery.

Blood glucose and lactate. Capillary glucose and lactate were measured every 30 min during recovery, as described above.

Additional measurements during the recovery period. HR was measured during exercise and the 5-h recovery period (Polar Pro trainer 5; Kempele, Finland). Data are presented as means of the first 2 h and the last 3 h of recovery. Resting metabolism was measured after 4.5 h of recovery. $\dot{V}O_2$, RER, and HR were measured in a supine position over 10 min, and data for the last 5 min were used.

Intervention drinks during recovery. The recovery supplementation during the first 2 h following exhaustive exercise and biopsy procedures was either carbohydrate (CHO) or an isocaloric protein with carbohydrate (CHO+PROT) drink. These supplements were provided in a randomized order. Supplementation was given after the first

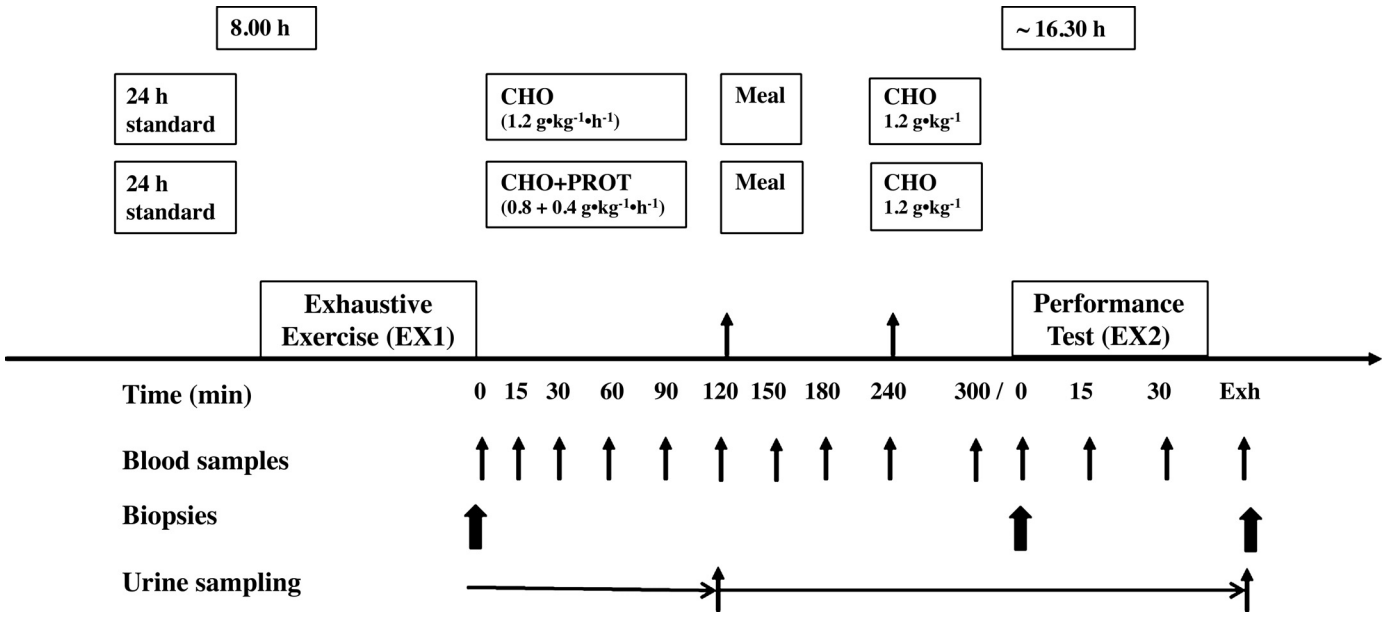


Fig. 1. Schematic overview of the test days with dietary interventions. The study was randomized, counterbalanced, and double-blinded. CHO, carbohydrate; CHO+PROT, carbohydrate and protein; EX1, exhaustive exercise before the dietary intervention; EX2, exercise performance test after the 5-h recovery.

biopsy, and again after 30, 60, and 90 min of recovery. Tissue and blood sampling were always completed before the participants drank any beverage.

CHO. The concentration of carbohydrates in CHO was 170 g/L (17%). The carbohydrate was a mixture of 85 g/L (50%) glucose and 85 g/L (50%) maltodextrin. Glucose was from Merck (Darmstadt, Germany), and the maltodextrin from WWR (Herlev, Denmark). Participants were given 0.6 g CHO/kg every 30 min during the first 90 min of recovery. Thus, 1.2 g CHO·kg^{-1}·hr^{-1} was ingested during the first 90 min of recovery.

CHO+PROT. The CHO+PROT drink was isocaloric with the CHO drink. The concentration of carbohydrate and protein was 113.7 and 56.3 g/L, respectively (170 g/L). Drinks consisted of 56.3 g/L glucose, 56.3 g/L maltodextrin, and 56.3 g/L whey protein. The protein was whey isolate protein (Lacprodan; SP-9225 Instant), provided by Arla Food Ingredients P/S (Aarhus, Denmark). Participants were given 0.4 g/kg of carbohydrate and 0.2 g/kg of whey protein every 30 min during the first 90 min of recovery. Thus, 0.8 g carbohydrate·kg^{-1}·h^{-1} and 0.4 g protein·kg^{-1}·h^{-1} were ingested during the first 90 min of recovery. All drinks were served in opaque bottles. To make the drinks comparable in taste, a noncaloric fruit flavored sweetener and 0.7 mg/L sodium chloride were added to the drinks.

Additional food and recovery supplementation. After 2 h of recovery, a meal containing minced meat, pasta, and tomato sauce was served according to body weight. The amounts of carbohydrate, protein, and fat served in the lunch were 1.7, 0.5, and 0.2 g/kg, respectively. After 4 h of recovery, a carbohydrate drink (1.2 g carbohydrate/kg) was provided after both CHO and CHO+PROT treatments.

Performance Test

The endurance performance test consisted of cycling until exhaustion at $W_{70\%}$ of $\dot{V}O_{2peak}$. After a standardized warm-up and 5 min of rest, participants started the performance test. Participants were blinded to time for the duration. $\dot{V}O_2$ and RER were measured over 90 s after 3.5 min every 15th min and during the last 60 s before exhaustion. After each $\dot{V}O_2$ and RER measurement, participants were asked for their rating of perceived exertion (RPE) followed by drawing blood samples for determination of lactate and glucose. During exercise, participants were asked to drink water approximately every 10 min. After the performance test, a third biopsy was taken from the same leg as the first biopsy.

Insulin

Plasma insulin concentrations were measured with an ELISA kit (Dako, Glostrup, Denmark).

Western Blot Analysis

Muscle homogenization. About 30 mg of muscle were freeze-dried with a Christ Alpha 1-2 LDplus freeze dryer (SciSqip, Shropshire, UK). Moisture was removed by suction for 2.5 h at a gas pressure of $\leq$0.04 mbar and air temperature of equal or less than -50°C. The samples were homogenized 1:100 in an ice-cold homogenizing buffer (pH 7.4), as previously described (26) with a Retsch MR400 mixer mill (Haan, Germany). The MR 400 was programmed to shake the muscles and buffer with a frequency of 30 Hz for three 30-s periods with 5 s between periods. After homogenization, homogenates were rotated for 60 min and centrifuged (11,500 *g* for 10 min at 4°C), and the protein concentration was measured. Samples were diluted and prepared for Western blot analysis, as previously described (26).

Primary antibodies. The following antibodies were obtained from Cell Signaling Technology (Beverly, MA): GS Ser^{641} (also called GS site 3a; no. 3891), AMPK Thr^{172} (no. 2531), PKB Ser^{473} (no. 9271), GSK-3 Ser^{21} (no. 9331), p70s6k Thr^{389} (no. 9205), AS160 Ser^{588} (no. 8930), TSC2 Thr^{1462}-Tuberin (no. 3611), and GAPDH-Clone 14C10 (no. 21185). Antibodies against AMPK-α_2 (sc-19131) and p70s6k Thr/Ser$^{421/424}$ (sc-7984-R) were obtained from Santa Cruz Biotechnology (Dallas, TX). Total GS and *site 2+2a* have been described and validated by Højlund et al. (19).

Glycogen Synthase Activity

Glycogen synthase (GS) activity was measured as described previously (20). In brief, duplicate measurements were performed in the presence of 0.01, 0.17, and 8 mM glucose-6-phosphate (G6P) in 96-well microtiter assay plates (Unifilter 350 plates; Whatman, Cambridge, UK).

Muscle Glycogen

Muscle glycogen was measured in two separate pieces of each biopsy. Muscle biopsies were freeze-dried, homogenized, and glycogen hydrolyzed before measurements of glucose units fluorometrically, as described previously (26).

Nitrogen Balance

Urine was collected in two fractions from the beginning of the initial glycogen-depleting exercise until 120 min of recovery, and from 120 min of recovery until the performance test was completed. Nitrogen balance was calculated on the basis of ingested proteins and nitrogen excretion in the urine. Urine nitrogen was analyzed with the Kjeldahl method (30). Total nitrogen excretion was calculated assuming 77.1% of total nitrogen loss via urine (61). Urea was measured with a QuantiChrom urea assay kit (DIUR-500 BioAssaySystem). Urine nitrogen concentration measured with the Kjeldahl method correlated with urea nitrogen concentration ($r = 0.996$; $P < 0.001$; $n = 34$).

Exclusion

Ten participants were included in the study. When the last five participants had completed the first dietary intervention, the power-control unit on the SRM ergometer failed to work. With the replacement power-control unit, heart rate was found to be ~20 beats lower and $\dot{V}O_2$ ~0.5 L/min less of predetermined load compared for the first of the last five participants tested. The data for this participant were then excluded from all analyses. For the last four participants, during their second dietary intervention exercise test, the load on the new SRM ergometer was adjusted to obtain comparable heart rate and VO_2, as occurred during their initial test with the original power-control. Therefore, the data on the performance test were excluded for these subjects, whereas data from blood and muscle samples were included.

Statistical analysis. Data are presented as means ± SE. Repeated-measures ANOVA was used to compare measurements during exercise and recovery periods, with a least significant difference (LSD) test used for post hoc testing. Student's paired *t* test was used to compare time to exhaustion and nitrogen balance after CHO+PROT and CHO. $P < 0.05$ was considered to be significant.

RESULTS

Fasted blood glucose was 4.8 ± 0.1, 5.1 ± 0.2, and 4.7 ± 0.1 mM before the baseline biopsy, CHO, and CHO+PROT interventions, respectively. Fasted blood lactate was 0.71 ± 0.08 and 0.88 ± 0.14 mM before CHO and CHO+PROT, respectively. Resting $\dot{V}O_2$, measured in a supine position was 228 ± 15 and 241 ± 15 ml O_2/min before CHO and CHO+PROT, respectively. RER values were 0.96 ± 0.03 and 0.96 ± 0.03 and HR was 49 ± 3 and 51 ± 3 beats/min before CHO and CHO+PROT, respectively. No differences in these resting measures were observed between trials ($P > 0.05$).

Table 1. *Oxygen uptake, RER, rating of perceived exertion, and heart rate during the exhaustive exercise prior to the dietary interventions*

	Arrival	5 min	30 min	50 min	Exhaustion	After Sprint
$\dot{V}O_2$, ml/min						
CHO	228 ± 15	2,813 ± 183	3,171 ± 106	3,209 ± 114	3,427 ± 137^T	
CHO+Prot	241 ± 15	2,990 ± 159	3,186 ± 136	3,339 ± 121	3,448 ± 105^T	
RER ($\dot{V}CO_2/\dot{V}O_2$)						
CHO	0.96 ± 0.03	0.94 ± 0.02	0.96 ± 0.01	0.95 ± 0.02	0.96 ± 0.02	
CHO+Prot	0.96 ± 0.03	0.92 ± 0.02	0.94 ± 0.01	0.95 ± 0.01	0.94 ± 0.01	
Blood glucose, mM						
CHO	5.1 ± 0.2	4.7 ± 0.1	4.6 ± 0.2	4.0 ± 0.1	3.3 ± 0.2	3.4 ± 0.2^T
CHO+Prot	4.7 ± 0.1	4.8 ± 0.1	4.7 ± 0.1	4.2 ± 0.2	3.8 ± 0.3	3.6 ± 0.3^T
Blood lactate, mM						
CHO		3.21 ± 0.43	2.69 ± 0.26	2.61 ± 0.32	3.01 ± 0.42	3.42 ± 0.62
CHO+Prot		3.10 ± 0.67	2.96 ± 0.67	3.04 ± 0.50	3.58 ± 0.47	4.42 ± 0.74
HR, beats/min						
CHO	49 ± 3	142 ± 6	158 ± 4	159 ± 4	166 ± 4	
CHO+Prot	51 ± 3	145 ± 6	160 ± 3	161 ± 3	167 ± 5	
RPE (Borg scale)						
CHO		11.3 ± 0.8	15.6 ± 0.5	15.8 ± 0.5	19.0 ± 0.3^T	
CHO+Prot		12.0 ± 0.8	15.1 ± 0.6	15.9 ± 0.7	19.1 ± 0.2^T	

Data are expressed as means ± SE; $n = 9$. Under "Arrival," data show resting measurements. CHO, carbohydrate; Prot, protein; HR, heart rate; RPE, rate of perceived exertion; ^{T}time effect using repeated-measures ANOVA. No significant differences were found in physiological responses during the exercise prior to the dietary interventions.

Exercise Prior to the Dietary Interventions

Time to exhaustion at $W_{70\%}$ was similar before the CHO and CHO+PROT interventions (107.0 ± 5.6 and 101.7 ± 9.0 min, respectively). During the exercise, $\dot{V}O_2$, heart rate, and RPE increased gradually (Table 1). Blood glucose concentration decreased gradually, whereas lactate increased rapidly and remained stable during exercise (Table 1). At exhaustion and after the 1-min sprints, blood glucose was ~3.5 mM (Table 1).

Recovery Period

Following exercise, blood glucose concentration increased rapidly after intake of CHO and CHO+PROT (Fig. 2*A*). Blood glucose was higher in CHO compared with CHO+PROT during the 90-min dietary intervention (treatment effect) and peaked at 9.1 ± 0.4 mM after 60 min. During the last 3 h of the recovery period, when diet was similar, blood glucose decreased (time effect), and there were no differences between conditions. Plasma insulin increased rapidly following intake of both CHO and CHO+PROT with no significant difference between interventions (Fig. 2*B*). Following exercise, blood lactate fell rapidly (Fig. 1*C*). HR was similar between CHO and CHO+PROT during the recovery period. After 4.5 h of recovery, resting $\dot{V}O_2$ (CHO: 307 ± 19 ml/min and CHO+PROT: 311 ± 18 ml/min), RER (CHO: 0.91 ± 0.03 in CHO+PROT 0.90 ± 0.02), and heart rate (CHO: 65 ± 3 and CHO+PROT: 65 ± 2 beats/min) were similar between CHO and CHO+PROT. Compared with the resting morning values, $\dot{V}O_2$ and HR were significantly elevated during the recovery period ($P < 0.01$).

Muscle Samples

Muscle glycogen concentration was 494.6 ± 29.1 mmol/kg dry wt in the baseline biopsy after an overnight fast (Fig. 3*A*). The exhaustive exercise bouts reduced the muscle glycogen stores to a similar degree with glycogen content being 141.9 ± 29.9 and 106.7 ± 30.7 mmol/kg dry wt before CHO and CHO+PROT supplementation, respectively (Fig. 3*A*). During recovery, rate of glycogen synthesis was 35.2 ± 3.8

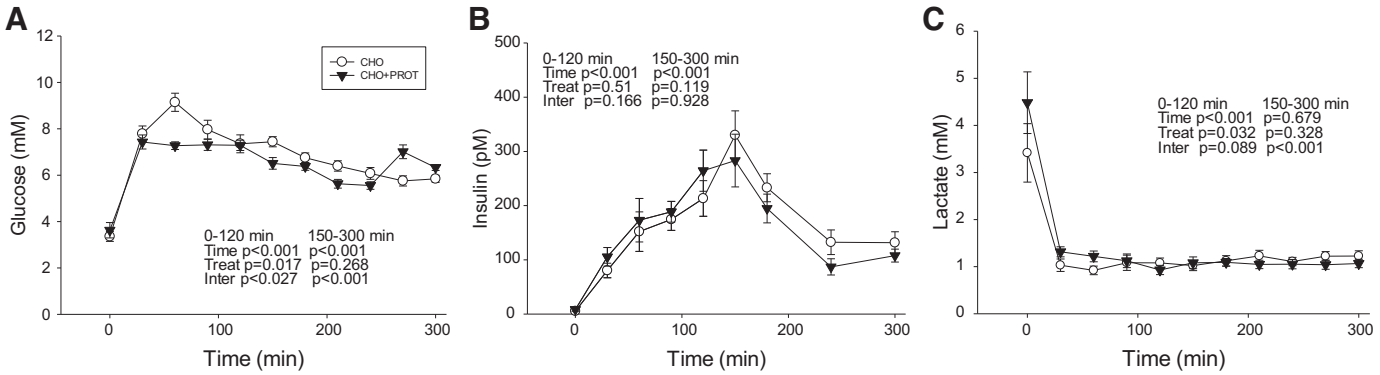


Fig. 2. Concentrations of glucose, insulin, and lactate in blood during the recovery period after intake of carbohydrate (CHO) or carbohydrate plus protein (CHO+PROT). Repeated-measures ANOVA was used for statistical analyses with time and diet (CHO vs. CHO+PROT) as treatment effects (Treat). Inter, interaction. Analyses were conducted separately during the dietary intervention period (0–120 min) and for the rest of the recovery period (150–300 min) when the diet was similar.

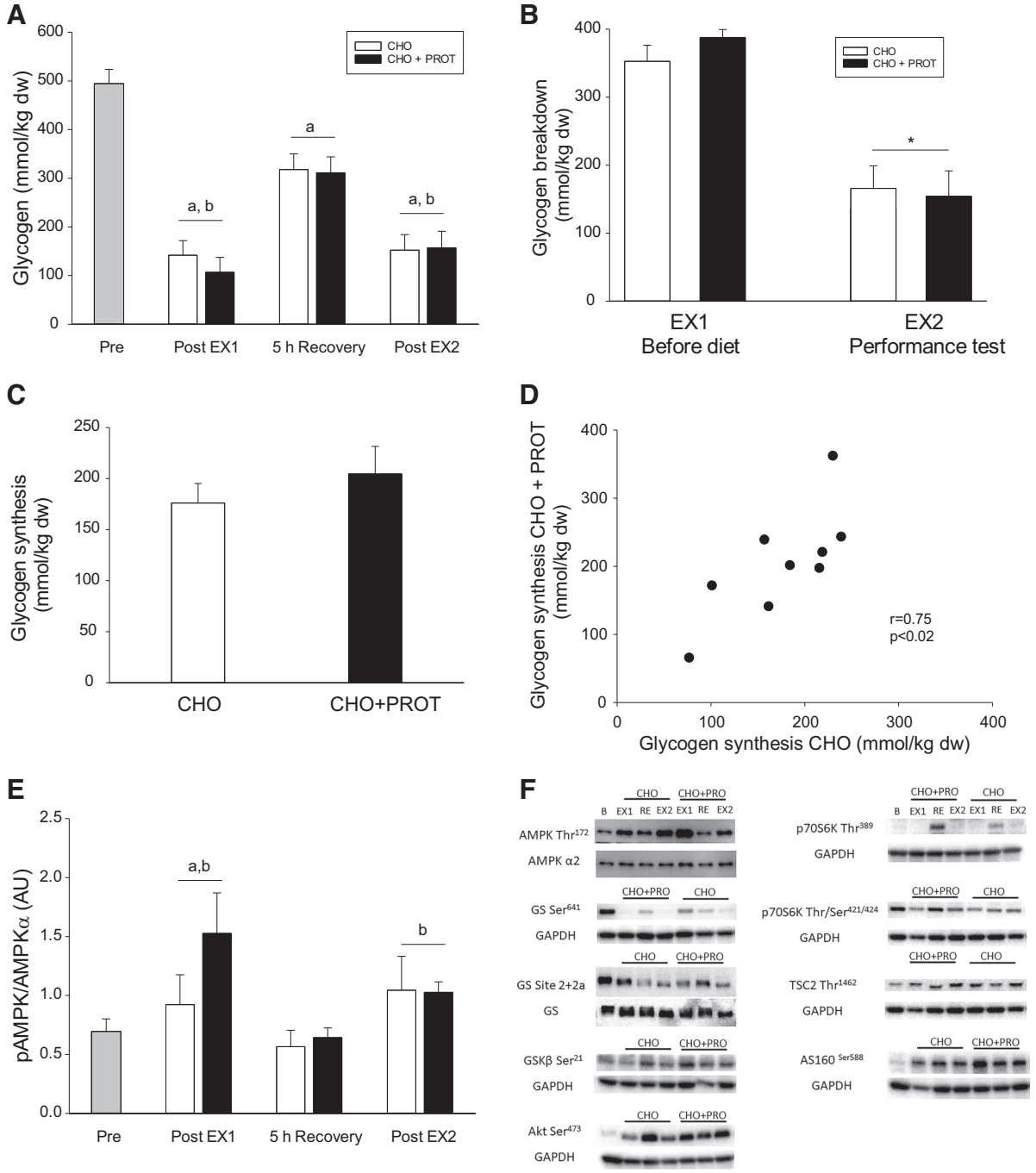
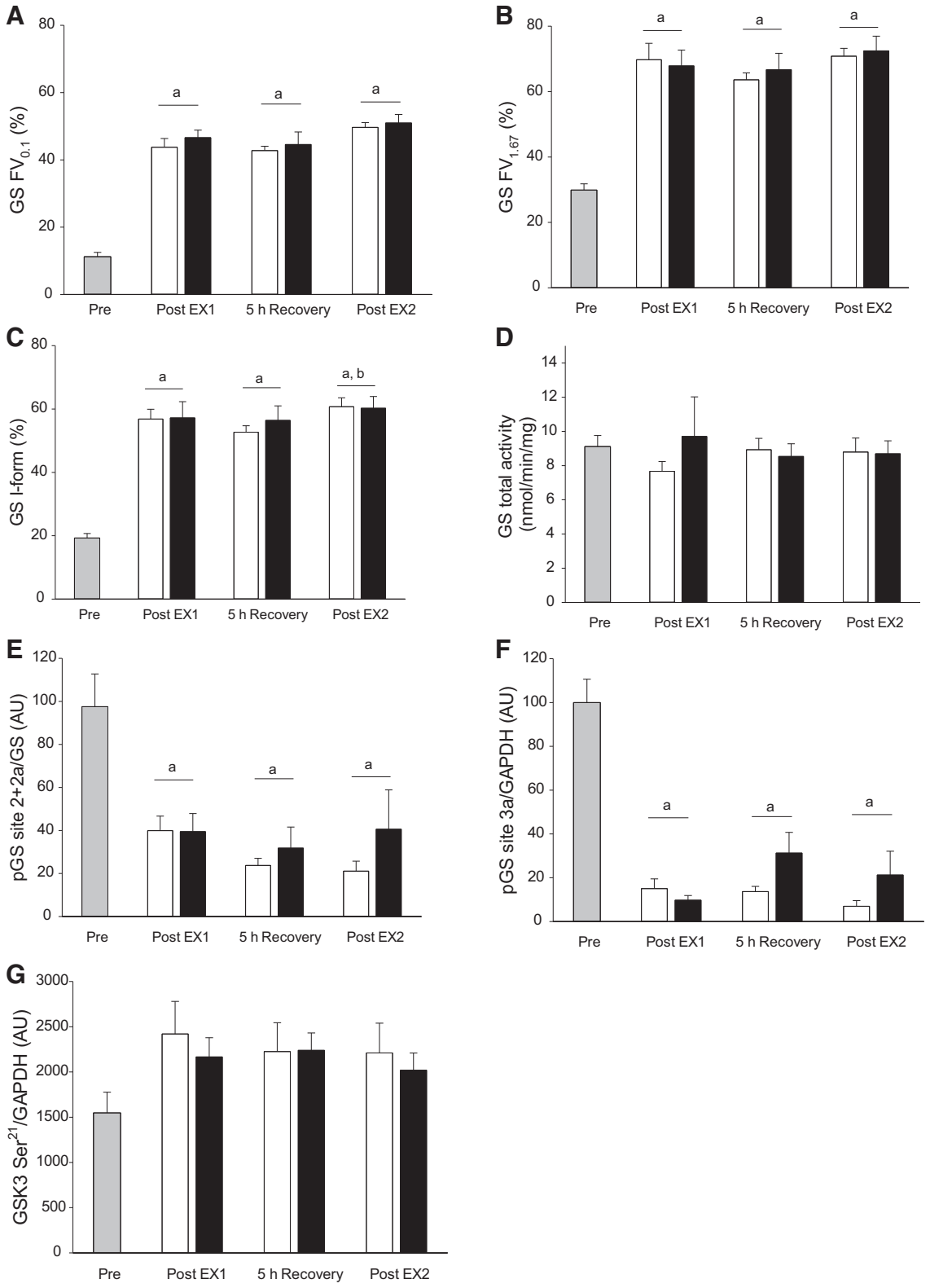


Fig. 3. Glycogen metabolism and phosphorylation of AMPK during the interventions. *A*: glycogen content in muscles before exercise, after the exercise before the dietary interventions, after a 5-h recovery and after the performance test. *B*: glycogen breakdown during the exercise before the dietary intervention and during the performance test. *C*: glycogen synthesis during the recovery period. *D*: correlation between glycogen synthesis during the 5-h recovery period with the two dietary interventions (CHO and CHO+PROT). *E*: AMPK Thr^{172} phosphorylation in muscles before exercise, after the exercise before the dietary interventions, after 5-h recovery and after the performance test. *F*: representative blots. Data are expressed as means ± SE; $n = 8$ or 9. See MATERIALS AND METHODS for description of the antibodies. *B*: basal before exercise; EX1, exercise before dietary intervention; RE, after 5-h recovery; EX2, performance test after the dietary intervention; CHO, carbohydrate intake during the first 2 h of recovery; CHO+PROT, carbohydrate plus protein during the first 2 h of recovery. $^{a}P < 0.05$, compared with Pre; $^{b}P < 0.05$, compared with 5-h recovery. $^{*}P < 0.05$ compared with EX1.

and 40.9 ± 5.4 mmol·kg dry wt^{-1}·h^{-1} in CHO and CHO+PROT, respectively (Fig. 3*C*; $P = 0.15$, Student's t test). Thus, before the performance test, glycogen content was 318.0 ± 32.1 mmol/kg dry wt for CHO and 311.3 ± 32.8 mmol/kg dry wt for CHO+PROT. Glycogen utilization during the exercise before dietary supplementation or during the subsequent performance tests did not differ between the two treatments (Fig. 3*B*). Rates of glycogen synthesis during the two treatments did not differ significantly (Fig. 3*C*). Rates of glycogen synthesis during the two treatments were significantly correlated (Fig. 3*D*; $r = 0.75$; $P < 0.02$). Phosphorylation of AMPK at Thr^{172} increased after both exercise sessions,

with no effect of the dietary treatments found (Fig. 3*E*). Glycogen synthase activity was investigated, and all parameters (GS $FV_{0.1}$, GS $FV_{1.67}$, and GS %-I-form) were activated after exercise to a similar degree before the dietary treatments (Fig. 4, *A–C*). Interestingly, GS remained activated during the 5-h recovery, despite a high rate of glycogen synthesis (Fig. 4, *A–C*). Phosphorylation of GS at *site 3* and *site 2+2a* was reduced after exercise and remained low during the 5-h recovery and agreed with the GS activity data. GS total activity was not influenced by the exercise or dietary treatments as expected



(Fig. 4, *E* and *F*). GSK-3 phosphorylation was not influenced by exercise or dietary intervention (Fig. 4*G*).

Phosphorylation of Akt at Ser^{473} increased immediately after exercise, and phosphorylation was elevated further after 5 h of recovery, but no differences were found between CHO and CHO+PROT (Fig. 5*A*). Phosphorylation of p70S6K at Thr^{389} was not increased immediately after exercise, but phosphorylation was elevated similarly in CHO and CHO+PROT after 5 h (Fig. 5*B*). Phosphorylation of p70S6K at Thr^{421}/Ser^{424} did not change during the dietary intervention (Fig. 5*C*). Likewise, TSC2 Thr^{1462} phosphorylation was unchanged at the time points studied (Fig. 5*D*). Phosphorylation of TBC1D4/AS160 Ser^{588} increased immediately after exercise and remained elevated to a similar level in CHO and CHO+PROT (Fig. 5*E*).

Performance Test

Time to exhaustion at $W_{70\%}$ lasted on average 8.4 ± 1.8 min longer in CHO+PROT than CHO (Fig. 6; $P < 0.009$; $n = 5$). Following the performance test protocol, muscle glycogen was reduced equally to 152.2 ± 32.0 and 157.1 ± 34.0 mmol/kg·dry wt in CHO and CHO+PROT, respectively (Fig. 3).

Data for $\dot{V}O_2$, RER, HR, and RPE from the performance test are summarized in Fig. 7. $\dot{V}O_2$, RER, heart rate, and RPE increased during the performance test, but there were no differences between the two dietary interventions. Blood glucose declined during the first minute of exercise, but returned to basal level at exhaustion (Fig. 7*C*). Lactate increased to ~2 mM during exercise, and there were no differences between CHO and CHO+PROT (Fig. 7*D*).

Nitrogen balance. Net nitrogen balance was positive in CHO+PROT (19.6 ± 7.6 mg/kg; $P = 0.04$). Nitrogen balance was not significantly different from zero in CHO (−10.7 ± 6.3 mg/kg; $P = 0.22$) but significantly lower than CHO+PROT ($P = 0.009$). Nitrogen excretion was higher in CHO+PROT than in CHO (9.9 ± 0.5 vs. 7.4 ± 0.6 g; $P = 0.008$) during the recovery period. Urea nitrogen accounted for 92.2 ± 1.3 and 91.9 ± 0.8% of total urine nitrogen excretion in CHO and CHO+PROT, respectively.

DISCUSSION

The present study is one of the first to obtain muscle biopsies that show endurance performance is improved after coingestion of protein and carbohydrate compared with intake of carbohydrate only during the first 2 h of the recovery period. The rate of glycogen synthesis and activation of anabolic signaling molecules during the recovery period were not noticeably different between CHO and CHO+PROT. Although performance improved after coingestion of protein and carbohydrate compared with carbohydrate only, glycogen degradation and activation of signaling molecules during the performance tests were not significantly different between treatments. On the other hand, nitrogen balance was positive only after coingestion of protein and carbohydrate and may have contributed to optimizing recovery of performance after exhaustive exercise.

Several studies have reported improved performance after coingestion of protein and carbohydrate compared with carbohydrate only (4, 54, 58, 60, 66). However, other studies have not found improved performance after coingestion of protein and carbohydrate (5, 43, 53), and this discrepancy needs to be clarified. In the present study, participants cycled until exhaustion before the 90-min dietary interventions, which was similar to the protocol used in our two previous studies (54, 60). In these studies, we demonstrated that providing a protein plus carbohydrate supplement during the first 90 min of recovery significantly improved exercise performance 18 h later compared with providing carbohydrate only (54, 60). Therefore, we have now demonstrated that providing a protein plus carbohydrate supplement in the first hours of recovery from exhaustive endurance exercise results in a better exercise performance both 5 and 18 h later compared with providing carbohydrate only.

The molecular mechanisms for the beneficial effects of protein intake is unclear. To the best of our knowledge, only one previous study has taken muscle biopsies in a setting in which coingestion of protein and carbohydrate recovers performance better than carbohydrate only (12). Ferguson-Stegall et al. (12) found a similar rate of skeletal muscle glycogen synthesis (25–30 mmol/kg wet wt during 4 h) as in the present study, and like the present study, there was no difference in rate of glycogen synthesis between treatments. However, Ferguson-Stegall et al. did find phosphorylation of mTOR to be higher 45 min after exercise when protein plus carbohydrate (chocolate milk) was ingested compared with carbohydrate only.

As mentioned previously, we did not find a significant difference in rate of glycogen synthesis between the interventions, despite reports that coingestion of protein and carbohydrates increased the rate of glycogen synthesis more than after intake of carbohydrate only (5, 70). Indeed, not all studies report elevated rates of glycogen synthesis after exercise when protein and carbohydrates are coingested (28, 62). However, the rate of glycogen synthesis is difficult to study because of variation in glycogen content in muscle biopsies. In a study by Jentjens et al. (28), glycogen content after the exercise before CHO and CHO+PROT interventions were 106 ± 19 and 176 ± 31 mmol/kg dry wt, respectively, but rate of glycogen

Fig. 4. Glycogen synthase activity and phosphorylation during the intervention. *A*: glycogen synthase fractional activity in the presence of 0.1 mM UDP-glucose (GS $FV_{0.1}$) in muscles before exercise, after the exercise before the dietary interventions, after 5-h recovery and after the performance test. *B*: glycogen synthase fractional activity in the presence of 1.67 mM UDP-glucose (GS $FV_{1.67}$) in muscles before exercise, after the exercise before the dietary interventions, after a 5-h recovery and after the performance test. *C*: glycogen synthase I-form in muscles before exercise, after the exercise before the dietary interventions, after 5-h recovery and after the performance test. *D*: total glycogen synthase activity in muscles before exercise, after the exercise before the dietary interventions, after 5-h recovery and after the performance test. *E*: glycogen synthase phosphorylation at *site 2+2a* in muscles before exercise, after the exercise before the dietary interventions, after 5-h recovery and after the performance test. *F*: glycogen synthase phosphorylation at *site 3* in muscles before exercise, after the exercise before the dietary interventions, after 5-h recovery and after the performance test. *G*: GSK3β Ser^{21} phosphorylation in muscles before exercise, after the exercise before the dietary interventions, after 5-h recovery and after the performance test. See Fig. 3 for blots. Data are expressed as means ± SE; $n = 8$ or 9. GS, glycogen synthase; GS $FV_{0.1}$, fractional activity with 0.1 mM UDP-glucose in assay buffer; GS $FV_{1.67}$, fractional activity with 1.67 mM UDP-glucose in assay buffer. GS I-form, fractional activity with 1.67 mM UDP-glucose and 0.01 mM glucose 6-phosphate; pGS, glycogen synthase phosphorylation. [a]$P < 0.05$ compared with Pre. [b]$P < 0.05$ compared with 5-h recovery.

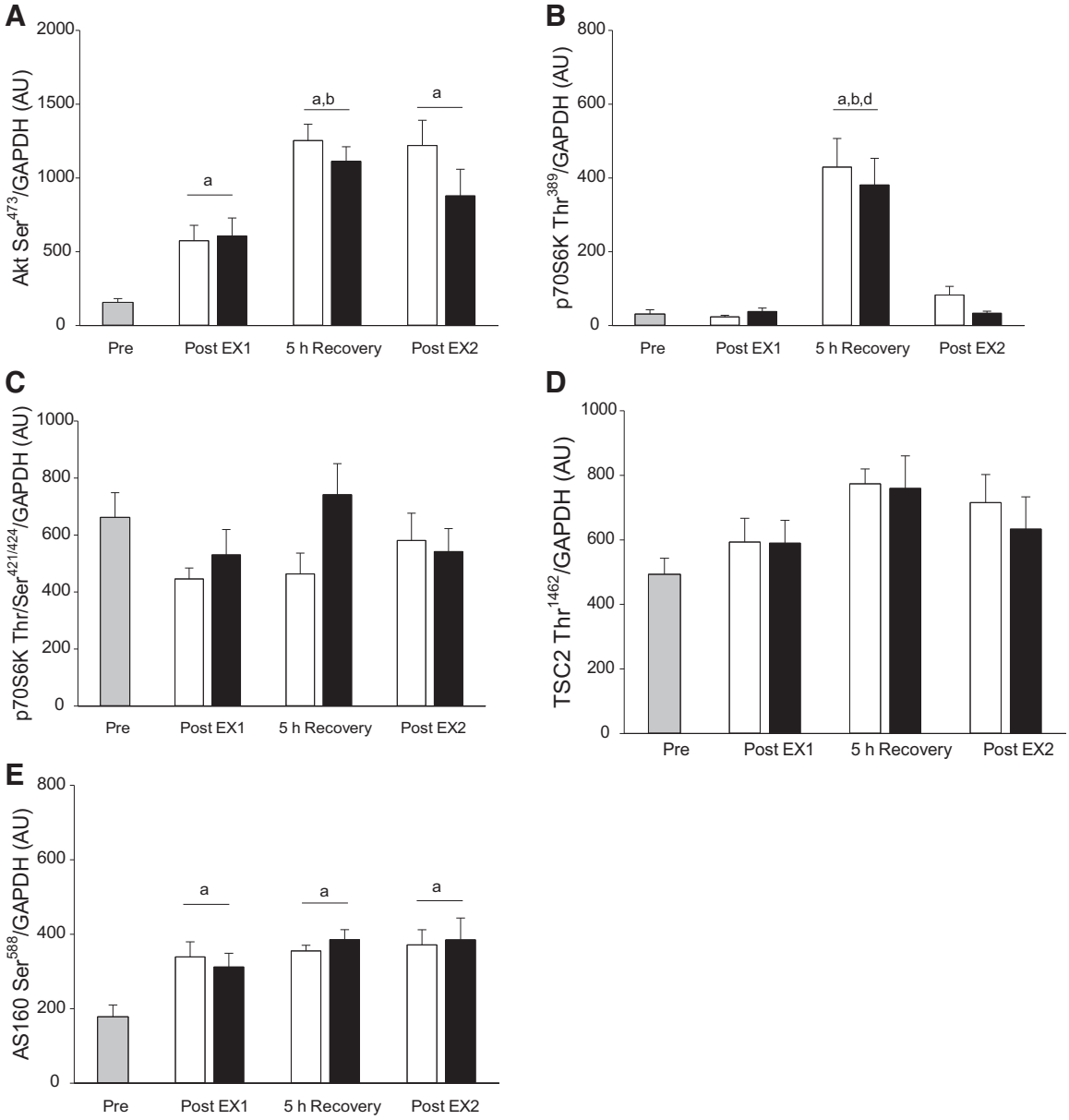


Fig. 5. Phosphorylation of signaling molecules during the interventions. Muscle biopsies were taken before exercise, after the exercise before the dietary interventions, after 5-h recovery and after the performance test for measurement of Akt Ser^{473} (*A*), p70S6K Thr^{389} (*B*), p70S6K $Thr/Ser^{421/424}$ (*C*), TCS2 Thr^{1462} (*D*), and AS160 Ser^{588} (*E*) phosphorylation. See Fig. 3 for blots. Data are expressed as means ± SE; $n = 8$ or 9. See MATERIALS AND METHODS for full names of proteins. $^{a}P < 0.05$ compared with Pre; $^{b}P < 0.05$ compared with 5-h recovery; $^{d}P < 0.05$ compared post-EX2.

synthesis was numerically lower in CHO than CHO+PROT (225 ± 22 vs 252 ± 48 mmol/kg dry wt). This is surprising, because low glycogen content activates glycogen synthase and stimulates glycogen synthesis (24, 33–35).

In the present study, glycogen content was 142 ± 30 and 107 ± 31 mmol/kg dry wt before CHO and CHO+PROT, respectively, and the rate of glycogen synthesis was numerically higher after CHO+PROT compared with CHO ($P = 0.15$; two-tailed *t* test). However, this tendency for a higher rate of glycogen synthesis in CHO+PROT could have been influenced by the numerically lower postexercise glycogen content in CHO+PROT compared with CHO (25). Moreover, rates of glycogen synthesis during the two dietary interventions (CHO+PROT and CHO) were significantly correlated ($r = 0.75$; $P < 0.02$; Fig. 3*D*), which suggests that interindividual variation (genetic or training status) determined the rate of glycogen synthesis rather than the treatment provided.

It has previously been reported that trained participants have higher rates of glycogen synthesis than untrained (13, 17). However, there was no significant correlation between $\dot{V}O_{2max}$ and rate of glycogen synthesis, suggesting that the rate of glycogen synthesis was not influenced by differences in training status. The rate of muscle glycogen synthesis has also been found to correlate with GLUT4 expression (13, 17). We did not measure GLUT4 expression but instead measured phosphorylation of AS160 (TBC1D1/TBC1D4), because increased phosphorylation improves insulin sensitivity and GLUT4 translocation to the sarcolemma (32). AS160 Ser^{588} phosphorylation

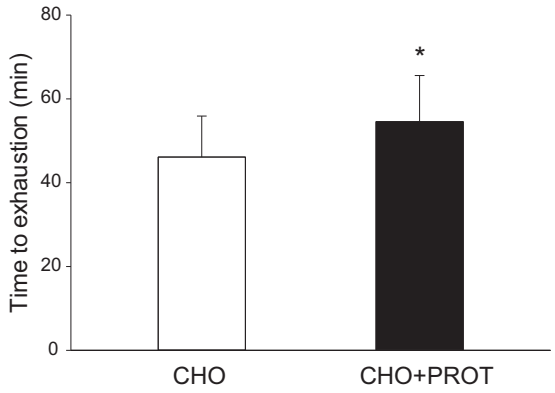


Fig. 6. Performance time to exhaustion after intake of CHO+PROT or CHO during the first 2 h of the 5-h recovery period. Data are expressed as means ± SE; $n = 5$. *$P < 0.05$ compared with CHO.

did not correlate with rate of glycogen synthesis, and AS160 Ser^{588} phosphorylation was similar after intake of CHO and CHO+PROT.

To summarize, CHO+PROT supplementation did not appear to enhance the rate of muscle glycogen synthesis during exercise recovery relative to the rate produced by an isocaloric CHO supplement. Furthermore, glycogen content was similar before the performance test after intake of CHO and CHO+PROT. Therefore, these results support the findings of Ferguson-Stegall et al. (12) that an improvement in exercise performance following CHO+PROT supplementation is not due to a higher muscle glycogen content, resulting from a more rapid recovery of muscle glycogen postexercise.

Hypoglycemia can also result in fatigue during prolonged endurance exercise (10). However, we observed no signs of hypoglycemia during the performance test after the dietary interventions, and the decline in blood glucose was more pronounced during the exhaustive exercise before the dietary interventions. This supports our previous findings that mechanisms other than blood glucose availability limits performance after recovery from exhaustive exercise (54). Importantly, the glycogen content at exhaustion was similar in the exercise session before and after the dietary interventions and independent of treatment, indicating that low glycogen content contributed to fatigue in all conditions. The fact that the increase in AMPK phosphorylation did not differ between treatments or the two exercise sessions is not surprising because the participants cycled until exhaustion, and similar metabolic stress may have developed and caused fatigue (31, 67).

Anabolic signaling was elevated in the recovery period, but there were no differences in activation of anabolic signaling between CHO and CHO+PROT. However, it is important to note that we did not take biopsies in the timeframe of protein supplementation, and the anabolic effect of protein intake lasts less than 5 h (3). The participants were biopsied seven times in total, and our priorities were obtaining the pre and post samples after the two bouts of exhaustive exercise. In the present study, phosphorylation of Akt was elevated immediately after exercise, agreeing with some (8, 64), but not all, studies (38–40, 68). It is, however, important to recognize that phosphorylation of Akt after muscle contractions is low compared with after insulin stimulation and not accompanied by phosphorylation of p70S6K at Thr^{389} (64). After the 5-h recovery, insulin concentration was elevated, and phosphorylation of Akt increased further and was paralleled by increased phosphorylation of p70S6K at Thr^{389}. However, there were no differences in the phosphorylation of these enzymes between CHO and CHO+PROT, which may be due to the similar insulin responses during these two dietary interventions. Moreover, TSC phosphorylation at Thr^{1462} was unchanged at the end of recovery in both CHO and CHO+PROT. Although many studies have shown that protein intake after exercise elevates phosphorylation of mTOR, p70S6K, and other signaling molecules that stimulate protein synthesis (42, 46, 49, 51, 56, 65), it is important to keep in mind that phosphorylation of p70S6K and mTOR are poor predictors of rate of protein synthesis (41). In addition, endurance exercise seems to stimulate myofibrillar or mitochondrial protein in skeletal muscle independently of mTORC1 activation (45).

In the present study, nitrogen balance was positive in CHO+PROT with intake of 1.3 g protein/kg during the recovery period. By contrast, nitrogen balance was negative for CHO, during which only 0.5 g protein/kg was ingested during the 5-h recovery. Endurance athletes require 1.6–2.0 g/kg of protein daily to maintain nitrogen balance (29, 61). It is important to note, that participants were studied after an overnight fast, and the protein intake during the 5-h recovery was the only protein intake during 15–18 h. The negative nitrogen balance after CHO indicates a catabolic state in the skeletal muscle and may explain the reduced performance following this intervention (50, 54, 60). The protein intake during the first 2 h of recovery for CHO+PROT was substantially higher than the dose required to maximally stimulate muscle protein synthesis (49), and urinary excretion of nitrogen was higher after coingestion of CHO+PROT than CHO, indicating that part of the ingested protein was metabolized. Therefore, the negative nitrogen balance after intake of CHO alone may be critical to detecting improved performance after CHO+PROT (50, 54, 60).

A most important finding in the present study was that glycogen synthase fractional activity remained highly activated without any decline during the 5-h recovery. High glycogen content normally inhibits GS activity (11, 24), and the finding that ~200 mmol/kg glycogen can be synthesized without any reduction in GS activity is remarkable. In this regard, muscle contraction increases GS activity via dephosphorylation of GS at *site 3* and *site 2+2a* (33, 34). In the present study, phosphorylation of GS at *site 3a* and *site 2+2a* remained low during the 5-h recovery, which explained the high activity. GSK3, which phosphorylates GS at the three sites, was not regulated by exercise, as expected (68). Exercise-induced activation of GS requires the protein phosphatase-1 binding subunit R_{GL} (*PPP1R3A*), suggesting that activation of protein phosphatase is required for exercise to activate GS (2). Recently, we showed that glycogen synthase activity was higher than expected for the glycogen content established 24 h after exercise (18). We can now expand this finding by showing that GS remains activated despite substantial glycogen synthesis during the first 5 h of recovery. Our recent study showed that AMPK is required for glycogen supercompensation (18). Although AMPK phosphorylation was not elevated after 5 h of recovery, our recent study showed that the $\alpha_1\beta_2\gamma_1$ subunit activity remained elevated even after AMPK phosphorylation had returned to basal level (18). Viewed comprehensively,

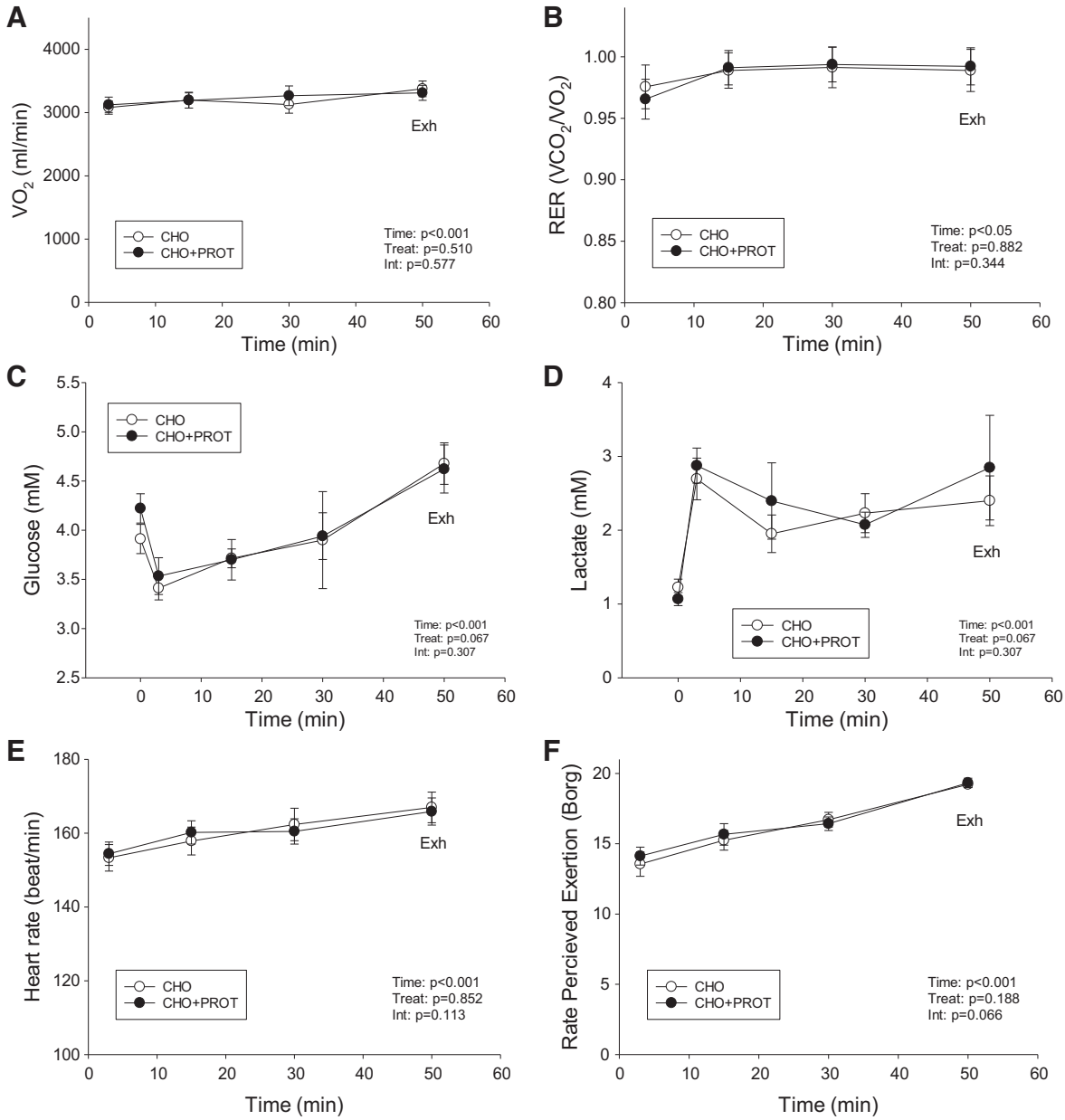


Fig. 7. Oxygen uptake, respiratory exchange ratio, glucose, lactate, heart rate, and perceived exertion during the endurance performance test after 5-h recovery. Data are expressed as means ± SE; $n = 9$. Ratings of perceived exertion and heart rate are shown during the exhaustive exercise prior to the dietary interventions.

these results suggest that GS activity can become decoupled from glycogen-mediated inhibition and may help explain how glycogen content can supercompensate after exhaustive exercise (7, 18).

It is a limitation of the present study that no muscle biopsies were taken during the first part of the dietary intervention when either protein and carbohydrate or carbohydrate only was supplied. This may explain why no significant differences in activation of anabolic signaling were observed between treatments. The small sample size, in particular, for performance, but our power analysis indicated the participant number was adequate. Finally, the study only included well-trained males, which preclude generalization of the results.

In conclusion, intake of CHO+PROT during the first 90 min after exhaustive exercise recovered endurance performance better than intake of carbohydrate only despite similar rates of glycogen synthesis during the recovery period. Although performance improved after coingestion of protein and carbohydrate, glycogen degradation and activation of signaling molecules during the performance tests were similar for both dietary interventions. Nitrogen balance was positive only after coingestion of protein and carbohydrate, suggesting that differences in protein synthesis during recovery may have contributed to the difference in exercise performance between the CHO+PROT and CHO treatments.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

M.A.D., P.B.J., T.I.-H., J.L.I., K.O., and J.J. conceived and designed research; M.A.D., J.L.A., P.B.J., J.B.B., T.I.-H., J.F.W., K.O., and J.J. performed experiments; M.A.D., J.L.A., P.B.J., J.B.B., E.I.J., B.S.S., J.F.W., K.O., and J.J. analyzed data; M.A.D., J.L.A., P.B.J., J.B.B., E.I.J., B.S.S., J.L.I., J.F.W., K.O., and J.J. interpreted results of experiments; J.L.A., J.B.B., and J.J. prepared figures; M.A.D. and J.J. drafted manuscript; M.A.D., J.L.A., P.B.J., J.B.B., E.I.J., T.I.-H., M.H., B.S.S., J.L.I., J.F.W., K.O., and J.J. edited and revised manuscript; M.A.D., J.L.A., P.B.J., E.I.J., T.I.-H., M.H., B.S.S., J.L.I., J.F.W., K.O., and J.J. approved final version of manuscript.

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