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High rates of muscle glycogen resynthesis after exhaustive exercise when carbohydrate is coingested with caffeine

David J. Pedersen, Sarah J. Lessard, Vernon G. Coffey, Emmanuel G. Churchley, Andrew M. Wootton, They Ng, Matthew J. Watt, and John A. Hawley

School of Medical Sciences, RMIT University, Bundoora, Victoria, Australia

Pedersen DJ, Lessard SJ, Coffey VG, Churchley EG, Wootton AM, Ng T, Watt MJ, Hawley JA. High rates of muscle glycogen resynthesis after exhaustive exercise when carbohydrate is coingested with caffeine. *J Appl Physiol* 105: 7–13, 2008. First published May 8, 2008; doi:10.1152/japplphysiol.01121.2007.—We determined the effect of coingestion of caffeine (Caff) with carbohydrate (CHO) on rates of muscle glycogen resynthesis during recovery from exhaustive exercise in seven trained subjects who completed two experimental trials in a randomized, double-blind crossover design. The evening before an experiment subjects performed intermittent exhaustive cycling and then consumed a low-CHO meal. The next morning subjects rode until volitional fatigue. On completion of this ride subjects consumed either CHO [4 g/kg body mass (BM)] or the same amount of CHO + Caff (8 mg/kg BM) during 4 h of passive recovery. Muscle biopsies and blood samples were taken at regular intervals throughout recovery. Muscle glycogen levels were similar at exhaustion [~75 mmol/kg dry wt (dw)] and increased by a similar amount (~80%) after 1 h of recovery (133 ± 37.8 vs. 149 ± 48 mmol/kg dw for CHO and Caff, respectively). After 4 h of recovery Caff resulted in higher glycogen accumulation (313 ± 69 vs. 234 ± 50 mmol/kg dw, $P < 0.001$). Accordingly, the overall rate of resynthesis for the 4-h recovery period was 66% higher in Caff compared with CHO (57.7 ± 18.5 vs. 38.0 ± 7.7 mmol·kg dw^{-1}·h^{-1}, $P < 0.05$). After 1 h of recovery plasma Caff levels had increased to 31 ± 11 μM ($P < 0.001$) and at the end of the recovery reached 77 ± 11 μM ($P < 0.001$) with Caff. Phosphorylation of CaMK^{Thr286} was similar after exercise and after 1 h of recovery, but after 4 h CaMK^{Thr286} phosphorylation was higher in Caff than CHO ($P < 0.05$). Phosphorylation of AMP-activated protein kinase (AMPK)^{Thr172} and Akt^{Ser473} was similar for both treatments at all time points. We provide the first evidence that in trained subjects coingestion of large amounts of Caff (8 mg/kg BM) with CHO has an additive effect on rates of postexercise muscle glycogen accumulation compared with consumption of CHO alone.

Akt; AMP-activated protein kinase; Ca^{2+}/calmodulin-dependent kinase

IT IS WELL ACCEPTED that the rate of muscle glycogen accumulation following glycogen-depleting exercise is enhanced by the provision of exogenous carbohydrate (CHO) (for review see Refs. 18, 21). In this regard, the dose, timing, and frequency of CHO administration have major roles in determining the rate and amount of glycogen resynthesized throughout the postexercise recovery period (17, 18). CHO ingestion alone, however, is not the only factor that alters glucose availability and hence glycogen resynthesis during recovery from exercise. The ingestion of caffeine, for example, has a negative effect on glucose metabolism (12, 13, 33). Caffeine ingestion before either an oral glucose tolerance test or a hyperinsulinemic-euglycemic clamp results in significant impairments in insulin-mediated glucose disposal and CHO storage compared with when no caffeine is ingested (11, 12).

While caffeine ingestion exerts a negative effect on skeletal muscle glucose disposal in resting humans, exercise appears to diminish such effects. The coingestion of caffeine with CHO during exercise (35) and exercise before caffeine ingestion (1) increase glucose availability. Yeo et al. (35) demonstrated that compared with glucose alone, caffeine [5 mg/kg body mass (BM)] coingested with CHO increased muscle glucose oxidation during 2 h of submaximal cycling. Battram et al. (1) recently reported that caffeine (6 mg/kg BM) ingested by moderately trained subjects during 90 min of glycogen-depleting exercise does not affect the rate of glycogen accumulation when large amounts of CHO (~400 g) are consumed during 5 h of recovery. Clearly, exercise/contraction exerts a positive and prolonged effect on muscle glucose uptake, and this is likely to be an important consideration when assessing the effects of caffeine on glucoregulatory mechanisms, particularly in well-trained athletes after exercise-induced glycogen depletion. Furthermore, the results of Battram et al. (1) strongly suggest that the factors driving muscle glycogen synthesis after exercise (i.e., insulin-independent mechanisms, low levels of glycogen at exhaustion, high insulin and glucose concentrations, training status of subjects) may override some of the potentially negative effects of caffeine observed on glucose metabolism at rest in untrained individuals (11, 12). Given the variable results of studies that have examined the effects of caffeine intake on muscle glycogen synthesis (9, 10, 28), we measured rates of muscle glycogen accumulation in highly trained individuals throughout 4 h of recovery from an exhaustive exercise depletion protocol during which subjects ingested caffeine in association with a CHO ingestion regimen that conforms to current sports nutrition guidelines (4). In addition, we determined a number of signaling proteins with putative roles in skeletal muscle glucose transport.

METHODS

Subjects

Seven endurance-trained cyclists/triathletes who were cycling >250 km/wk (12–15 h/wk) volunteered to participate in this study. The subjects' age, BM, peak O$_2$ uptake ($\dot{V}O_{2peak}$), and peak power output (PPO) were 26.9 ± 5.6 yr, 71.79 ± 11.8 kg, 60.0 ± 3.7 kg/min, and 329 ± 35 W (values are means $\pm$ SD). Before participation all experimental procedures and possible risks of the study were explained to each subject before written informed consent was given.

Address for reprint requests and other correspondence: J. A. Hawley, School of Medical Sciences, RMIT Univ., PO Box 71, Bundoora 3083, Victoria, Australia (e-mail: john.hawley@rmit.edu.au).

The study protocol was approved by the RMIT University Human Research Ethics Committee.

Study Overview

This study comprised a randomized, double-blind crossover design. Subjects completed two trials with appropriate diet and exercise control before each experiment (described below). Subjects were not habitual caffeine users, and they did not consume caffeine-containing substances (coffee, chocolate, and soft drinks) for 48 h before each trial. Diet and exercise diaries were used to standardize food intake and physical activity for 48 h before an experiment and to verify compliance. The evening before an experimental trial, subjects reported to the laboratory to perform a bout of intermittent exhaustive cycling. After the glycogen-depleting ride, subjects were provided with a low-carbohydrate diet. The next morning subjects reported to the lab and rode until volitional fatigue at a power output corresponding to 70% $\dot{V}_{O_{2peak}}$. On completion of this ride, subjects either consumed CHO or CHO plus caffeine during 4 h of passive recovery. Muscle biopsies and blood samples were also taken at regular intervals throughout the recovery period.

Preliminary Testing

On their first visit to the laboratory, subjects performed a maximal incremental cycling test to volitional fatigue on a Lode ergometer (Groningen, The Netherlands) for the determination of PPO and $\dot{V}_{O_{2peak}}$. The maximal incremental test protocol commenced at a workload equivalent to 3 W/kg BM, and the work rate was increased by 50 W after the first 150 s and then by 25 W every 150 s thereafter until exhaustion (14). Throughout the maximal test subjects breathed through a mouthpiece attached to a Quark b^2 metabolic cart (COSMED, Rome, Italy). Expired gas was passed through a flowmeter, an O_2 analyzer, and a CO_2 analyzer that were calibrated before testing with a 3-liter Hans-Rudolph syringe and gases of known concentration (4.00% CO_2 and 16.00% O_2). The flowmeter and gas analyzers were connected to a computer that calculated minute ventilation, oxygen uptake ($\dot{V}_{O_2}$), CO_2 production ($\dot{V}_{CO_2}$), and the respiratory exchange ratio (RER) from conventional equations. $\dot{V}_{O_{2peak}}$ was reported as the highest $\dot{V}_{O_2}$ for any 60 s. PPO was calculated by adding the work completed on the final workload to the last successfully completed workload, and this value was used to determine the appropriate power outputs for the experimental trials described below.

Dietary and Exercise Control

Muscle glycogen levels were lowered before each experimental trial by a combination of exhaustive cycle exercise and dietary intervention. The exercise protocol utilized to deplete muscle glycogen in our lab has been described in detail previously (27). About 14 h before each experimental trial, subjects reported to the laboratory and performed an intermittent ride to volitional fatigue. After a 5-min warm-up, subjects commenced cycling for 2 min at 90% of PPO, followed immediately by 2-min recovery at 50% of PPO. This work-recovery protocol was maintained until subjects were unable to complete 2 min of cycling at 90% PPO, determined as an inability to maintain a cadence of 60 rpm for 15 s. At this time the power output was lowered to 80% PPO with the same work-to-rest ratio. When subjects were unable to complete 2 min of high-intensity cycling, the power output was lowered to 70% of PPO and finally 60% PPO. Exercise was terminated when subjects could not complete 2 min of cycling at 60% of PPO. This protocol was chosen so as to maximally deplete both type I and type II muscle fibers of the subjects' muscle glycogen stores (25). During exercise, subjects received no feedback with respect to either the number of repetitions they had performed at each power output or the elapsed time. Water was consumed ad libitum, and an electric fan (wind speed 17 km/h) was positioned to increase air circulation and evaporative cooling. On completion of

exercise, subjects were provided with a low-carbohydrate diet (1.2 g/kg BM carbohydrate, 0.8 g/kg BM protein, and 1.4 g/kg BM fat) to be consumed as their evening meal. All diets were constructed by a sports dietician, and dietary control included the individualization of food plans for each subject (relative to BM) and food preferences. All food and drinks were supplied prepackaged to subjects. Subjects were also supplied with a food checklist to record their daily intake for 36 h leading into a trial day. The intent of the first prolonged, exhaustive exercise bout and the subsequent diet intervention was to ensure that on the morning of an experimental trial (described below), subjects commenced exercise in a glycogen-depleted state.

Experimental Trials

Subjects completed two experimental trials ~10 days apart, at the same time of day (commencing at ~0700) after an ~10- to 12-h overnight fast. On arrival at the lab, subjects voided and rested in a supine position for 10 min. A 20-gauge Teflon catheter (Terumo, Tokyo, Japan) was then inserted into a vein in the antecubital region of the arm for blood sampling, and a resting blood sample (~6 ml) was taken. The catheter was then flushed with ~1 ml of sterile saline (Astra Zeneca, North Ryde, Australia) to keep the catheter patent and sterile, a procedure that was completed after each subsequent blood draw. Local anesthesia [2–3 ml of 1% Xylocaine (lignocaine)] was administered to the skin, subcutaneous tissue, and fascia of the vastus lateralis, and three incisions were made in the same leg (~5 cm distal) in preparation for subsequent biopsies.

After 10 min subjects mounted the cycle ergometer and commenced cycling at 70% $\dot{V}_{O_{2peak}}$ until volitional fatigue. During these rides, water was consumed ad libitum. At the point of exhaustion with the subject still seated on the ergometer, a first muscle biopsy was taken (within 10 s) with a 6-mm Bergstrom needle modified with suction. Approximately 100–150 mg of muscle was removed, immediately frozen in liquid N_2, and stored at −80°C until analysis. A 6-ml blood sample was also taken at this time. Subjects then dismounted the ergometer and began 4 h of recovery, during which they rested in a supine position. Throughout recovery from one trial subjects consumed 4 g CHO/kg BM in the form of sports bars, gels, and CHO-containing sports drinks. CHO was consumed within 5 min of the cessation of exercise and again after 60, 120, and 180 min. During recovery from the other trial, the same CHO ingestion regimen was followed and a total of 8 mg/kg BM caffeine was administered in two equal doses immediately after exercise and after 2 h of recovery (Caff). Caffeine was added to a specially formulated CHO-containing sports drink (GlaxoSmithKline Consumer Healthcare). Muscle biopsies were taken after 60 and 240 min of recovery, while blood samples (6 ml) were taken at regular intervals (30, 60, 90, 120, 180, 240 min) throughout recovery. Laboratory conditions remained constant for all testing (21–22°C, 40–50% relative humidity).

Analytical Procedures

Blood glucose and insulin. One milliliter of whole blood was immediately analyzed for glucose concentration with an automated glucose/lactate analyzer (YSI 2300, Yellow Springs, Ohio). Five milliliters of whole blood was placed into a tube containing fluoride EDTA, mixed, and centrifuged at 4,000 rpm for 8 min at 0°C. The plasma was stored at −80°C for later analysis of plasma insulin concentration by radioimmunoassay (insulin RIA/ELISA, LINCO Research, St. Charles, MO).

Plasma caffeine concentrations. Plasma caffeine concentration was determined with a HPLC technique, according to the methods of Cox et al. (6). Caffeine and β-hydroxyethyltheophylline were purchased from Sigma Chemical (St. Louis, MO). Aqueous HPLC solvent was prepared by using water obtained from a Milli-Q water purification system from Millipore (Bedford, MA). Ammonia buffer (pH 9) was prepared by the addition of ammonia to a saturated ammonium chloride solution. For the extraction of caffeine in plasma, 1 ml of

plasma in a 10-ml screw-capped plastic centrifuge tube was added to 1 ml of 0.15 M Ba(OH)$_2$. The tube was then vortexed for 1 min, 1 ml of 5% zinc sulfate was added, and the tube was vortexed for another 1 min. After protein precipitation, the sample was centrifuged at 4,000 rpm for 5 min. The upper layer was transferred to a Wassermann tube, where 100 mg of NaCl, 50 μl of internal standard (β-hydroxyethyltheophylline, 100 μg/ml), and 100 μl of ammonia buffer were added. Extraction was executed with the addition of 5 ml CHCl$_3$-MeOH (9:1 vol/vol) followed by vortexing for 2 min and centrifugation at 4,000 rpm for 5 min. The organic (lower) layer was isolated and passed through a Pasteur pipette containing anhydrous Na$_2$SO$_4$. The extract was evaporated to dryness under a stream of N$_2$ in a 40°C heating block. The residue was reconstituted in 250 μl of HPLC eluant, and 25 μl was injected onto the HPLC system. The concentration range for the standard curve was 2.4–30 μg/ml. HPLC analysis of caffeine in plasma was performed with a Waters analytic HPLC system (Waters, Milford, MA). This consisted of a Waters model 600E Powerline quaternary solvent delivery system and a Waters WISP 717 Plus autoinjector. The samples were separated at room temperature on an Adsorbosphere HS C$_{18}$ column (5 μm, 4.6-mm ID × 150 mm; Alltech Associates, Deerfield, IL) with an Adsorbosphere C$_{18}$ guard column, (5 μm, 4.6-mm ID × 7.5 mm). The mobile phase used for the separation was 10 mM KH$_2$PO$_4$-acetonitrile (91:9 vol/vol), and the flow rate was 2.0 ml/min. The peaks were determined with a Waters 484 tunable absorbance detector at 256 nm. The data were processed with the Waters Millennium 2010 multisystem software data analysis system. Ratio of internal standard-to-caffeine peak heights was used for quantification, and the retention time was 5.2 min for caffeine elution and 3.8 min for the internal standard β-hydroxyethyltheophylline. None of the subjects was able to guess the experimental treatment received during the two trials.

Muscle glycogen and metabolites. Muscle samples were divided into several fractions. One fraction was freeze-dried, dissected free of all nonmuscle contaminants under magnification, and powdered. One aliquot (~4 mg) of freeze-dried muscle was extracted, incubated with 250 μl of 2 M hydrochloric acid at 100°C for 2 h, and then neutralized with 750 μl of 0.667 M sodium hydroxide for subsequent determination of glycogen concentration via enzymatic analyses with fluorometric detection (26). Glycogen concentration was expressed as millimoles of glycogen per kilogram of dry weight (dw). The net rate of glycogen resynthesis was calculated from the differences observed between various time points. A second aliquot of freeze-dried muscle (~3 mg) was used for the determination of muscle phosphocreatine (PCr), ATP, creatine, and lactate levels by fluorometric techniques (26). Free ADP and AMP concentrations were calculated with the assumption of equilibrium of the adenylate kinase and creatine kinase reactions (8). Free ADP was calculated with the measured ATP, creatine, and PCr values, an estimated H$^+$ concentration (32), and the creatine kinase equilibrium constant of 1.66 × 10^9. Free AMP concentration was calculated from the estimated free ADP and the measured ATP with the adenylate kinase constant of 1.05.

Western blots. Muscle samples were homogenized in ice-cold homogenization buffer containing 50 mM Tris·HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 50 mM NaF, 5 mM Na pyrophosphate, 1 mM DTT, 10 μg/ml trypsin inhibitor, 2 μg/ml aprotinin, 1 mM benzamidine, and 1 mM phenylmethylsulfonyl fluoride. The homogenate was then centrifuged, and the supernatant was collected and aliquoted for determination of protein concentration (Pierce, Rockford, IL). Aliquots of lysate (80 μg of protein) were resuspended in Laemmli sample buffer, resolved by SDS-PAGE on 12% polyacrylamide gels, transferred to nitrocellulose membranes, blocked with 5% nonfat milk, washed with TBST (10 mM Tris·HCl, 100 mM NaCl, 0.02% Tween 20), and incubated with 10 ml of appropriate primary antibody overnight at 4°C. Membranes were washed with TBST and incubated with an appropriate secondary antibody. Proteins were visualized by chemiluminescence and quantified by densitometry. The amount of phosphorylated proteins of the densitometric quantification is expressed as arbitrary units. Polyclonal anti-phospho-CaMK II (no. 3361, Cell Signaling Technology, Danvers, MA) and monoclonal anti-phospho-Akt^{Ser473} (no. 4058, Vell Signaling) were used. Anti-phospho-AMP-activated protein kinase (AMPK)^{Thr172} was raised against AMPK α-peptide (KDGEFLRpTSCGAPNY) as described previously (5). Anti-rabbit secondary antibody and enhanced chemiluminescence reagents were from Amersham Biosciences (Little Chalfont, UK) and Pierce Biotechnology.

Statistical analysis. Data were analyzed by two-way analysis of variance (ANOVA) with repeated measures (SigmaStat for Windows version 3.11). Where significance was detected, post hoc analysis was performed with the Student-Newman-Keuls test. All values are expressed as means and SD, with the critical level of significance established at $P < 0.05$.

RESULTS

Diet and Exercise Compliance

Analysis of exercise and food diet diaries confirmed that subjects were compliant with both requirements in the 48 h before an experimental trial. There were no differences in exercise time to exhaustion between the two experimental trials (57 min 43 s ± 15 min 30 s vs. 60 min 30 s ± 14 min for CHO and Caff, respectively).

Blood Glucose, Insulin, and Caffeine Concentrations

Table 1 displays blood glucose and plasma insulin concentrations at rest and during recovery from exhaustive exercise. There was no difference in blood glucose concentrations at rest or immediately after exercise. Blood glucose levels increased within 30 min of carbohydrate ingestion after cessation of exercise in both CHO and Caff (~70%, $P < 0.001$) and remained elevated throughout the 4-h recovery period compared with values at exhaustion. Blood glucose concentration gradually decreased after 60–90 min of recovery in CHO ($P < 0.05$) but not in Caff (Table 1). Indeed, compared with the

Table 1. *Blood glucose and plasma insulin concentrations at rest and after exercise*

	Rest	0 h	0.5 h	1 h	1.5 h	2 h	3 h	4 h
Glucose, mmol/l								
CHO	3.9±0.2	3.5±0.3	6.1±0.8[a,b]	5.9±0.8[a,b]	5.4±1.1[a,b]	5.7±1.2[a,b]	4.7±1.0[b,c,d,e]	4.6±0.6[b,c,d,e]
Caff	4.0±0.3	3.6±0.6	6.1±0.5[a,b]	6.3±0.8[a,b]	6.0±1.1[a,b,f]	5.6±1.4[a,b]	5.2±0.9[a,b,f]	5.2±0.9[a,b,f]
Insulin, μU/ml								
CHO	8.3±3.2	4.3±1.7	24.4±11.7	24.5±9.3	35.0±10.9[g,h]	34.0±15.7[g,h]	44.3±21.3[g,h]	36.8±24.5[g,h]
Caff	9.4±3.3	4.2±1.9	30.2±15.2[h]	29.2±7.8[h]	46.3±16.7[g,h]	46.0±20[g,h]	68.5±32.5[i]	46.9±30.4[g,h]

Values are means ± SD. During recovery subjects consumed 1 g carbohydrate/kg body mass (BM) (CHO) or 1 g carbohydrate/kg BM + 8 mg caffeine/kg BM (Caff). Glucose significant difference ($P < 0.05$): [a]vs. rest, [b]vs. 0 h, [c]vs. 0.5 h, [d]vs. 1 h, [e]vs. 2 h, [f]Caff vs. CHO. Insulin significant difference ($P < 0.05$): [g]vs. rest, [h]vs. 0 h, [i]vs. all.

ingestion of CHO alone, coingestion of caffeine with CHO resulted in higher blood glucose levels after 3 h and 4 h of recovery ($P < 0.05$ compared with exhaustion). After CHO ingestion, insulin concentration increased in both CHO and Caff treatments after 60–90 min of recovery ($P < 0.05$) and thereafter remained higher than at exhaustion throughout the remainder of the recovery period for both treatments ($P < 0.05$, Table 1). Plasma insulin levels were consistently elevated (20–50%) throughout the last 2–3 h of recovery in Caff such that the area under the plasma insulin vs. time curve was significantly greater for Caff than for CHO ($P < 0.05$). Caffeine was undetectable in the samples taken before and throughout the CHO trial. However, the coingestion of caffeine with CHO immediately after exercise and again after 2 h of recovery resulted in marked increases in plasma caffeine concentrations. After 1 h plasma caffeine levels had increased to 31 ± 11 µM ($P < 0.001$), and at the end of the recovery period they reached 77 ± 11 µM ($P < 0.001$).

Muscle Metabolites

Table 2 displays the muscle metabolite concentrations immediately after exercise and during the recovery period. ATP levels were similar between treatments and throughout recovery. PCr values increased during recovery, but there were no differences in the rate of resynthesis between treatments. Free AMP concentrations were lower after 1 and 4 h of recovery, but there were no differences in between CHO and Caff treatments.

Muscle Glycogen Concentrations

At exhaustion, muscle glycogen levels were markedly depleted to ~80 mmol/kg dw, with no differences observed between the two trials (74 ± 55 vs. 76 ± 21 mmol/kg dw for CHO and Caff, respectively; Fig. 1). After 1 h of recovery, muscle glycogen content was increased by a similar amount (~80%) in both trials (133 ± 38 vs. 149 ± 48 mmol/kg dw for CHO and Caff, respectively). Accordingly, the net rate of synthesis over this period was 59 ± 28 vs. 58 ± 31 mmol·kg dw^{-1}·h^{-1} for CHO and Caff, respectively. After 4 h of recovery the coingestion of caffeine with CHO resulted in greater glycogen accumulation (313 ± 69 vs. 234 ± 50 mmol/kg dw, $P < 0.001$). Accordingly, the overall rate of resynthesis for the 4-h recovery period was higher in Caff

Table 2. *Postexercise muscle metabolite concentrations*

	0 h	1 h	4 h
ATP, mmol/kg dw			
CHO	25.4±1.9	24.0±2.1	26.1±2.8
Caff	25.4±3.2	25.4±2.4	24.2±3.9
PCr, mmol/kg dw			
CHO	72.1±15.5	84.3±11.9*	84.6±8.9*
Caff	73.7±11.8	85.4±10.8*	84.0±12.7*
Free AMP, µmol/kg dw			
CHO	0.82±0.45	0.35±0.22*	0.36±0.13*
Caff	0.69±0.37	0.37±0.18	0.34±0.14*

Values are means ± SD. During recovery subjects consumed 1 g carbohydrate/kg BM (CHO) or 1 g carbohydrate/kg BM + 8 mg caffeine/kg BM (Caff). PCr, phosphocreatine; dw, dry weight. *Significantly different ($P < 0.05$) vs. 0 h within treatment.
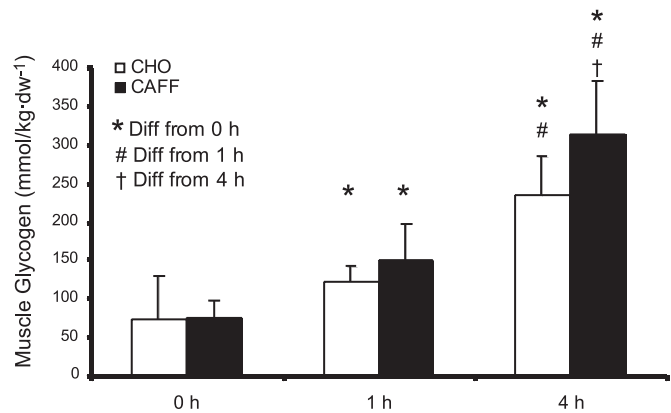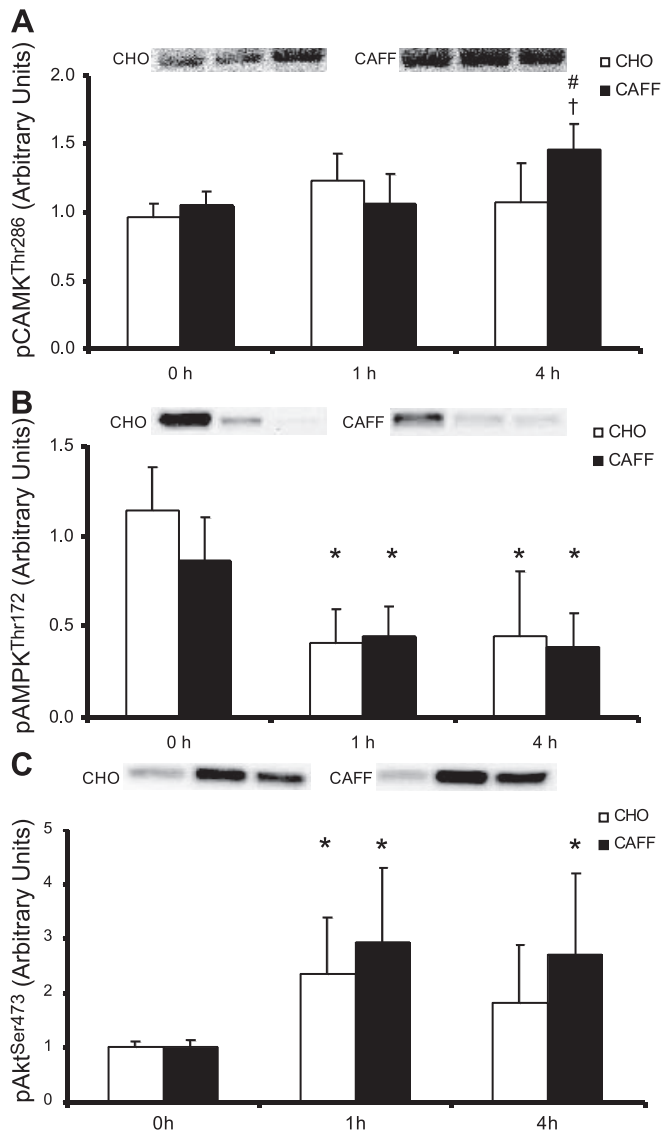

Fig. 1. Skeletal muscle glycogen content immediately after exercise (0 h) and after 1 h and 4 h of recovery following cycling to volitional fatigue [~70% peak O$_2$ uptake ($\dot{V}$O$_{2peak}$)]. During recovery subjects consumed 1 g carbohydrate/kg body mass (BM) (CHO) or 1 g carbohydrate/kg BM + 8 mg caffeine/kg BM (Caff). *Significant difference vs. 0 h; #significant difference vs. 1 h; †significant difference, CHO vs. Caff 4 h ($P < 0.05$). All values are means + SD.

compared with CHO (57.7 ± 18.5 vs. 38.0 ± 7.7 mmol·kg dw^{-1}·h^{-1}, $P < 0.05$).

Protein Kinases

Phosphorylation of CaMK^{Thr286} did not differ within or between trials immediately after exercise or after 1 h of recovery (Fig. 2A). However, after 4 h of recovery CaMK^{Thr286} phosphorylation was higher in Caff compared with CHO ($P < 0.05$). Phosphorylation of AMPK^{Thr172} was similar for CHO and Caff immediately after exercise and was lower after 1 and 4 h of recovery for both treatments (Fig. 2B, $P < 0.05$). Akt^{Ser473} phosphorylation was similar in both trials after exercise and increased during recovery in both trials ($P < 0.05$). After 1 h of recovery phosphorylation of Akt^{Ser473} tended to be higher in Caff than in CHO ($P = 0.06$).

DISCUSSION

The novel finding of the present investigation was that the coingestion of caffeine with CHO after exhaustive exercise resulted in significantly greater accumulation of muscle glycogen after 4 h of recovery compared with consumption of CHO alone. The overall rate of resynthesis in the present investigation (~60 mmol·kg dw^{-1}·h^{-1}) is the highest reported for human subjects under real-life conditions. While Bergstrom and Hultman (2) found rates of synthesis >80 mmol·kg dw^{-1}·h^{-1}, such values were obtained after intravenous glucose and fructose infusion.

Glycogen synthesis rates have been studied extensively with a variety of exercise protocols and postexercise dietary regimens (for review see Ref. 21). Although the highest rates of resynthesis (40–45 mmol·kg dw^{-1}·h^{-1}) have been reported when large (1.0–1.8 g·kg^{-1}·h^{-1}) amounts of CHO have been consumed at frequent (15–60 min) intervals, it should be noted that at any given rate of CHO intake large variability exists in the rate of glycogen synthesis. This is likely due to differences in the training status of subjects, the type and frequency of CHO administration, the mode of CHO administration (i.e., oral intake vs. clamped infusion), as well as the duration of time over which muscle glycogen synthesis rates are calcu-

Fig. 2. Skeletal muscle CaMK^{Thr286} (*A*), AMP-activated protein kinase (AMPK)^{Thr172} (*B*), and Akt^{Ser473} (*C*) phosphorylation immediately after exercise (0 h) and after 1 h and 4 h of recovery following cycling to volitional fatigue ($\sim$70% $\dot{V}O_{2peak}$). During recovery subjects consumed 1 g carbohydrate/kg BM (CHO) or 1 g carbohydrate/kg BM + 8 mg caffeine/kg BM (Caff). *Significant difference vs. 0 h; #significant difference vs. 1 h; †significant difference, CHO vs. caffeine 4 h ($P < 0.05$). All values are means + SD.

synthase activity. However, Thong et al. (33) previously reported that despite decreases in both glycogen synthase activity (17% fractional velocity of glycogen synthase and 35% I form) and leg glucose uptake (50%) after exhaustive one-legged knee extensor exercise and caffeine ingestion (5 mg/kg BM), rates of muscle glycogen accumulation following a hyperinsulinemic-euglycemic clamp (100 μU/ml) were similar ($\sim$300 mmol glucosyl units/kg dw after 100 min). Furthermore, Rush and Spriet (31) demonstrated that physiological doses of caffeine that elicited concentrations similar to those observed in the present study (50–100 μM) inhibited glycogen phosphorylase *a* activity, a condition that would be expected to favor rather than impede glycogen resynthesis.

The time course of glycogen resynthesis occurs in two distinct phases: an early (0–60 min) "insulin-independent" phase in which the rates of glycogen accumulation are high followed by a late (1–48 h) "insulin-dependent" phase in which the rate of glycogen resynthesis is somewhat lower (19, 30). Somewhat surprisingly, most studies that have employed a CHO ingestion protocol and determined glycogen content from biopsy samples have not measured glycogen resynthesis until after 2 h of recovery. An exception was the recent investigation of Battram et al. (1) in which biopsies were taken after just 30 min of recovery during which caffeine and CHO were ingested. In that study, resynthesis rates following caffeine plus CHO ingestion were 72 mmol·kg dw^{-1}·h^{-1} for 30 min of recovery and then declined during the subsequent 90 min and remained constant during the remainder of the 5-h recovery period such that overall the net rate of resynthesis was $\sim$50 mmol·kg dw^{-1}·h^{-1}. In contrast to these findings, our results reveal that rates of muscle glycogen synthesis were similar ($\sim$60 mmol·kg dw^{-1}·h^{-1}) after 1 h of recovery for both treatments, but remained elevated with the coingestion of caffeine and CHO throughout the 4-h observation period. Of note was the finding that the high rates of glycogen synthesis sustained during the later stages of recovery coincided with both higher blood glucose and insulin levels after the coingestion of caffeine with CHO (Table 1). Whether caffeine-induced adrenaline release stimulated hepatic glucose output or whether caffeine promotes intestinal absorption as has been previously suggested (35) cannot be determined in the present investigation. However, the insulinotropic effects of caffeine have been previously noted (29). While we did not determine plasma free fatty acid (FFA) levels in the present investigation, Battram et al. (1), using a similar exercise regimen and caffeine ingestion protocol, previously reported elevations in serum FFA levels throughout 5 h of recovery. In that study (1), the caffeine-induced increases in FFA concentration declined as insulin levels increased in response to frequent CHO ingestion throughout recovery.

Exercise and insulin stimulate glucose transport by separate pathways, and their maximal effects on muscle glucose uptake are additive (15). While it is generally accepted that increases in cytosolic Ca^{2+} mediate the effect of muscle contractions on glucose transport (16), activation of AMPK has also been deemed necessary for contraction-stimulated glucose transport into skeletal muscle (10, 22, 28). Although the precise mechanism(s) by which AMPK stimulates glucose transport is currently unknown, it is believed that activation of this kinase results in the phosphorylation of unknown target proteins, which leads to the translocation of GLUT protein to the plasma

lated. In the present study we specifically selected highly trained subjects, an exhaustive exercise depletion protocol that mimicked race conditions, and a CHO ingestion regimen that conforms to current sports nutrition guidelines (4) so that the results would be applicable to "real-world" situations. The rates of glycogen resynthesis after CHO alone was consumed ($\sim$40 mmol·kg dw^{-1}·h^{-1}) were in excellent agreement with results from previous studies using similar ingestion regimens. However, the high rates of glycogen accumulation when caffeine was coingested with CHO ($\sim$60 mmol·kg dw^{-1}·h^{-1}) strongly suggest that the factors driving glycogen accumulation after exercise override caffeine's previously reported negative effects on glucose metabolism seen during resting conditions in untrained individuals (12, 23, 33). A limitation of the present investigation is the lack of measurement of muscle glycogen

membrane (10). In an effort to identify a possible mechanism for the greater rates of muscle glycogen accumulation after the coingestion of caffeine with CHO during recovery, we measured several signaling proteins considered to play putative roles in glucose transport into skeletal muscle. We observed that phosphorylation of CaMK^{Thr286} was similar between trials immediately after exercise and also after 1 h of recovery, the latter being the time period during which rates of glycogen resynthesis were also the same (Fig. 2A). However, at the end of the 4-h recovery period CaMK^{Thr286} phosphorylation was significantly higher when caffeine was coingested with CHO than when CHO was ingested alone (Fig. 2A). While the recovery period from 2 to 4 h in the caffeine trial coincided with greater rates of glycogen resynthesis (and thus presumably glucose transport into muscle), without additional biopsies we cannot determine whether this outcome was associated with increased signaling to glucose transport through CaMK.

We observed a reduction in the exercise-induced phosphorylation of AMPK^{Thr172} after 1 h of recovery that was similar for both ingestion protocols (Fig. 2B) and that coincided with comparable rates of glycogen resynthesis (and presumably muscle glucose transport) as well as the recovery of muscle metabolites (Table 2). However, during the latter stages of recovery at a time when rates of muscle glycogen resynthesis were substantially higher after the coingestion of caffeine with CHO, AMPK phosphorylation remained similar between treatments (Fig. 2B). While our results do not support a role for AMPK in caffeine-induced glucose transport, Jensen et al. (20) recently reported that AMPK is causally linked to the caffeine-stimulated glucose uptake response. These workers provide evidence to demonstrate that caffeine exerts an isoform-specific increase in AMPK α_1 activity and acetyl-CoA carboxylase (ACC)β phosphorylation in the absence of total AMPK^{Thr172} phosphorylation (20). Accordingly, they propose that investigations that have only measured total AMPK^{Thr172} phosphorylation may be prone to type 2 errors and have resulted in false conclusions with regard to the effect of caffeine on AMPK activation. Other studies (34) have also suggested that caffeine-induced increases in Ca^{2+} signaling (CaMK) and AMPK activity are both involved in mediating the increase in muscle glucose transport. However, both of these investigations (18, 29) utilized pharmacological rather than physiological doses of caffeine, and caution should be used when comparing their results to those of the present investigation.

In addition to a role for Ca^{2+} and AMPK in glucose transport, protein kinase B/Akt has also been implicated as an important link between insulin signaling cascades and mechanisms important for GLUT4 translocation (for review see Ref. 24). Akt is a Ser/Thr kinase with three isoforms that are all expressed in skeletal muscle. The time course of contraction-stimulated glucose transport and the activation of Akt/protein kinase B are similar, raising the possibility that Akt may function in signaling to glucose transport in the working muscle. Glycogen synthase kinase (GSK)3 is a physiological target of Akt: phosphorylation of GSK3 decreases its activity toward glycogen synthase, which leads to increased glycogen synthesis (9). Isoform-specific silencing of Akt isoforms reveals that Akt2 and, to a lesser extent, Akt1 play essential roles in insulin-stimulated GLUT4 translocation and glucose transport (3). We observed a robust increase in the phosphorylation of Akt^{Ser473} with the cessation of exercise for both treatment

conditions (Fig. 2C). The increase tended to be higher after the ingestion of caffeine with CHO after both 1 and 4 h of recovery, but failed to reach statistical significance. Akt seems to regulate glucose uptake by phosphorylating and inhibiting the Rab-GTPase-activating protein AS160. Thus it is tempting to speculate on the role of Akt in glucose transport given that the Akt substrate AS160 has been identified as an important regulator of GLUT4 traffic. We have recently shown that AS160 is phosphorylated in human skeletal muscle after endurance exercise with concomitant phosphorylation of Akt (7), providing correlative evidence to suggest AS160 is an exercise-responsive protein with a role in glucose uptake. However, further work will be needed to test the hypothesis that caffeine induces an increase in Akt signaling to AS160 and leads to increased glucose transport into skeletal muscle.

In conclusion, the results from the present investigation provide the first evidence that after a bout of glycogen-depleting exercise caffeine coingested with CHO has an additive effect on rates of postexercise muscle glycogen accumulation. Part of this effect may be due to the higher blood glucose and insulin concentrations observed after the coingestion of CHO with caffeine, compared with ingestion of CHO alone. The overall (4 h) rate of resynthesis observed in the present investigation ($\sim$60 mmol·kg dw^{-1}·h^{-1}) with caffeine ingestion is, to the best of our knowledge, the highest reported for human subjects under physiological conditions. Whether lower doses of caffeine can increase postexercise glycogen resynthesis rates to the same extent remains to be determined.

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