HIGH RATES OF MUSCLE GLYCOGEN RESYNTHESIS AFTER EXHAUSTIVE EXERCISE WHEN CARBOHYDRATE IS CO-INGESTED WITH CAFFEINE

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ABSTRACT

We determined the effects of the co-ingestion of caffeine with carbohydrate on rates of muscle glycogen resynthesis during recovery from exhaustive exercise in 7 trained subjects who completed two experimental trials in a randomized, double-blind crossover design. The evening prior to an experiment subjects performed intermittent exhaustive cycling and then consumed a low-carbohydrate meal. The following morning subjects reported to the lab and rode until volitional fatigue. Upon completion of this ride subjects consumed either carbohydrate (CHO; 4 g.kg BM\(^{-1}\)) or the same amount of carbohydrate plus caffeine (CAFF, 8 mg.kg BM\(^{-1}\)) 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\(^{-1}\) d.w) and increased by a similar amount (~80%) after 1 h of recovery (133 ± 37.8 vs. 149 ± 48 mmol.kg\(^{-1}\) d.w. for CHO and CAFF respectively). After 4 h of recovery CAFF resulted in higher glycogen accumulation (313 ± 69 vs. 234 ± 50 mmol.kg\(^{-1}\) d.w, \(P<0.001\)). Accordingly the overall rate of resynthesis for the 4 h recovery period was 66% higher in CAFF compared to CHO (57.7 ± 18.5 vs. 38.0 ± 7.7 mmol.kg\(^{-1}\) d.w.h\(^{-1}\), \(P < 0.05\)). After 1 h of recovery plasma caffeine levels has 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\(^{\text{Thr286}}\) was similar post-exercise and after 1 h of recovery but after 4 h CAMK\(^{\text{Thr286}}\) phosphorylation was higher in CAFF than CHO (\(P<0.05\)). Phosphorylation of AMPK\(^{\text{Thr172}}\) and Akt\(^{\text{Ser473}}\) was similar for both treatments at all time points. In conclusion, we provide the first evidence that in trained subjects, the coingestion of large amounts of caffeine (8 mg.kg BM\(^{-1}\)) with carbohydrate has an additive effect on rates of post-exercise muscle glycogen accumulation compared to when carbohydrate alone is consumed.

Key words: Akt, AMPK, CAMK, exhaustive exercise.
INTRODUCTION

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 (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 post-exercise 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 to 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 co-ingestion of caffeine with CHO during exercise (35) and exercise prior to caffeine ingestion (1) increase glucose availability. Yeo et al. (35) demonstrated that compared with glucose alone, caffeine (5 mg·kg body mass [BM]⁻¹) co-ingested with CHO increased muscle glucose oxidation during 2 h of submaximal cycling. Battram et al. (1) have recently reported that caffeine (6 mg·kg BM⁻¹) 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 gluco-regulatory 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 post-
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 seen 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 hr of recovery from an exhaustive exercise-depletion protocol during which subjects ingested caffeine in association with a carbohydrate ingestion regimen that conforms to current sports nutrition guidelines (4). In addition, we determined a number of signalling proteins with putative roles in skeletal muscle glucose transport.

METHODS

Subjects

Seven endurance trained cyclists/triathletes who were cycling >250 km wk⁻¹ (12-15 h/wk) volunteered to participate in this study. The subjects age, BM, peak O₂ uptake (VO₂ peak) and peak power output (PPO) were 26.9 ± 5.6 yr, 71.79 ± 11.8 kg, 60.0 ± 3.7 ·kg·min⁻¹ and 329 ± 35 W (values are mean ± SD). Prior to participation all experimental procedures and possible risks of the study were explained to each subject before their written informed consent was given. 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 prior to each experiment (described subsequently). Subjects were not habitual caffeine users neither did they 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 prior to an experiment and to verify compliance. The evening prior to an experimental trial, subjects reported to the laboratory to perform a bout of intermittent exhaustive cycling. Following the glycogen-depleting ride, subjects were provided with a low-carbohydrate diet. The following morning subjects reported to the lab and rode until volitional fatigue at a power output corresponding to 70% VO2 peak. Upon completion of this ride, subjects either consumed CHO or carbohydrate 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 VO2peak. The maximal incremental test protocol commenced at a workload equivalent to 3 W·kg⁻¹ BM, and the workrate 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 b² metabolic cart (COSMED, Rome, Italy). Expired gas was passed through a flowmeter, an O₂ analyzer, and a CO₂ analyzer that were calibrated before testing using a 3-L Hans-Rudolph syringe and gases of known concentration (4.00% CO₂ and 16.00% O₂). The flowmeter and gas analyzers were connected to a computer that calculated minute ventilation, oxygen uptake (VO₂), CO₂ production (VCO₂), and the respiratory exchange ratio (RER) from conventional equations. VO₂ peak was reported as the highest VO₂ 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 subsequently described experimental trials.

*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 rev.min⁻¹ for 15 s. At this time the power output was lowered to 80% PPO with the same work: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 fibres 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, nor the elapsed time. Water was consumed *ad libitum* and an electric fan (wind speed 17 km.h⁻¹) positioned to increase air circulation and evaporative cooling. Upon completion of exercise, subjects were provided with a low- carbohydrate diet (1.2 g.kg BM⁻¹ carbohydrate, 0.8g.kg BM⁻¹ protein and 1.4 g.kg BM⁻¹ 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 pre-packaged 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 h) after a ~10-12 h overnight fast. Upon arrival at the lab, subjects voided and rested in a supine position for 10 min. Then a 20 gauge Teflon Catheter (Terumo, Tokyo, Japan) was 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 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 anaesthesia (2-3 mL of 1% Xylocaine (lignocaine)) was administered to the skin, subcutaneous tissue and fascia of the *vastus lateralis* and three incisions 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% VO$_2$ peak 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 sec) using 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$^{-1}$ in the form of
sports bars, gels and carbohydrate-containing sports drinks. Carbohydrate 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 carbohydrate ingestion regimen was followed and a total of 8 mg.kg BM⁻¹ caffeine administered in two equal doses immediately post exercise and after 2 h of recovery (CAFF). Caffeine was added to a specially formulated carbohydrate-containing sports drink (GlaxoSmithKline, Consumer Healthcare, U.K.) 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% RH).

Analytical procedures

**Blood glucose and insulin**

One mL of whole blood was immediately analyzed for glucose concentration using an automated glucose/lactate analyzer (YSI 2300, Yellow Springs, Ohio). Five mL of whole blood was placed into a tube containing fluoride EDTA, mixed and centrifuged at 4000 rev-min⁻¹ 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 Inc., St Charles, MO, U.S.A).

**Plasma caffeine concentrations**

Plasma caffeine concentration was determined using 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 0.15M Ba(OH)$_2$. The tube was then vortexed for 1 min, 1 ml of 5% zinc sulphate added and the tube 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$^{-1}$) 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 $^\circ$C heating block. The residue was reconstituted in 250 µL HPLC eluant, and 25 µL was injected onto the HPLC system. The concentration range for the standard curve was 2.4 – 30 µg.ml$^{-1}$. 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 x 150 mm; Alltech Associates, Deerfield, IL) with an adsorbosphere C$_{18}$ guard column, (5 µm, 4.6 mm ID x 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$^{-1}$. The peaks were determined with a Waters 484 tunable absorbance detector at 256 nm. The data were processed with Waters Millenium 2010 multisystem software data analysis system. Ratio of internal standard: caffeine peak heights were used for quantification, and the retention time for caffeine elution was 5.2 min, and for the internal standard β-hydroxyethyltheophylline it was 3.8 min. None of the subjects were able to guess the experimental treatment they 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 non-muscle contaminants under magnification and powdered. One aliquot (~4 mg) of freeze-dried muscle was extracted and with 250 µL of 2 M hydrochloric acid, incubated 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.kg d.w.⁻¹. 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, 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 using the measured ATP, creatine, and PCr values, an estimated H⁺ concentration (32), and the creatine kinase equilibrium constant of 1.66 x 10⁹. 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, 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 collected and aliquoted for determination of protein concentration (Pierce, Rockford, IL, U.S.A.). Aliquots of lysate (80 µg of protein) were re-suspended in Laemmli sample buffer, resolved by SDS-PAGE on 12% polyacrylamide gels, transferred to nitrocellulose membranes, blocked with 5% non-fat 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-CAMKII (#3361, Cell Signalling Technology, Danvers, MA) and monoclonal anti-phospho-Akt Ser473 (#4058, Veal Signalling). Anti-phospho-AMPK thr172 was raised against AMPK alpha peptide (KDGEFLRpTSCGAPNY) as described previously (5). Anti-rabbit secondary antibody and enhanced chemiluminescence reagents were from Amersham Biosciences (Buckinghamshire, U.K.) and Pierce Biotechnology (Rockford, IL, U.S.A.).

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 using the Student-Newman-Keuls test. All values are expressed as means and standard error (SE) 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 prior to an experimental trial. There were no differences in exercise time to exhaustion between the two experimental trials (57min 43s ± 15min 30s vs. 60min 30s ± 14min 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 following cessation of exercise in both CHO and CAFF (~70%, P <0.001) and remained elevated throughout the 4 h recovery period compared to 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 to the ingestion of carbohydrate alone, co-ingestion of caffeine with carbohydrate resulted in higher blood glucose levels after 3 h and 4 h of recovery (P <0.05 compared to exhaustion). Following 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 hr of recovery in CAFF such that the area under the plasma insulin versus time curve was significantly greater for CAFF than CHO (P<0.05). Caffeine was undetectable in the samples taken before and throughout the CHO trial. However, the co-ingestion of caffeine with carbohydrate immediately after exercise and again after 2 h of recovery resulted in marked increases in plasma caffeine concentrations. After 1 h plasma caffeine levels has 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\(^{-1}\) d.w, with no differences observed between the two trials (74 ± 55 vs. 76 ± 21 mmol·kg\(^{-1}\) d.w for CHO and CAFF respectively, Figure 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\(^{-1}\) d.w. for CHO and CAFF, respectively). Accordingly, the net rate of synthesis over this period was 59 ± 28 vs 58 ± 31 mmol·kg\(^{-1}\) d.w.hr for CHO and CAFF respectively. After 4 h of recovery the co-ingestion of caffeine with CHO resulted in greater glycogen accumulation (313 ± 69 vs. 234 ± 50 mmol·kg\(^{-1}\) d.w, P<0.001). Accordingly, the overall rate of resynthesis for the 4 h recovery period was higher in CAFF compared to CHO (57.7 ± 18.5 vs. 38.0 ± 7.7 mmol·kg\(^{-1}\) d.w.h\(^{-1}\), P < 0.05).

Protein Kinases

Phosphorylation of CAMK\(^{Thr286}\) did not differ within or between trials immediately post-exercise or following 1 h of recovery (Figure 2A). However, after 4 h of recovery CAMK\(^{Thr286}\) phosphorylation was higher in CAFF compared to CHO (P<0.05). Phosphorylation of AMPK\(^{Thr172}\) was similar for CHO and CAFF immediately post exercise and was lower after 1 and 4 h of recovery for both treatments (Figure 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 CHO (P=0.06).

DISCUSSION

The novel finding of the present investigation was that the co-ingestion of caffeine with carbohydrate after exhaustive exercise resulted in significantly greater accumulation of muscle glycogen after 4 h of recovery compared to when carbohydrate alone was consumed.
The overall rate of resynthesis in the present investigation (~60 mmol·kg⁻¹ d.w·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⁻¹ d.w·h⁻¹ such values were obtained after intravenous glucose and fructose infusion.

Glycogen synthesis rates have been studied extensively using a variety of exercise protocols and post-exercise dietary regimens (for review see (21)). Although the highest rates of resynthesis (40-45 mmol·kg⁻¹ d.w·h⁻¹) have been reported when large (1.0-1.8 g·kg⁻¹·h⁻¹) amounts of carbohydrate have been consumed at frequent (15-60 min) intervals, it should be noted that at any given rate of carbohydrate 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 carbohydrate administration, the mode of carbohydrate administration (i.e. oral intake versus clamped infusion), as well as the duration of time over which muscle glycogen synthesis rates are calculated. In the present study we specifically selected highly-trained subjects, an exhaustive exercise-depletion protocol that mimicked race conditions, and a carbohydrate 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 carbohydrate alone was consumed (~40 mmol·kg⁻¹ d.w·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 co-ingested with carbohydrate (~60 mmol·kg⁻¹ d.w·h⁻¹) strongly suggest that the factors driving glycogen accumulation post-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 synthase (GS) activity. However, Thong et al. (28) have previously reported that despite decreases in both GS
activity (17% fractional velocity of GS and 35% I form) and leg glucose uptake (50%) after exhaustive one-legged knee extensor exercise and caffeine ingestion (5 mg.kg⁻¹ BM), rates of muscle glycogen accumulation following a hyperinsulinemic-euglycemic clamp (100 µU/mL) were similar (~300 mmol glucosyl units·kg⁻¹ dry wt after 100 min). Furthermore, Rush and Spriet (31) have demonstrated that physiological does of caffeine that elicited similar concentrations to those observed in the present study (50-100 uM) inhibit glycogen phosphorylase a activity, a condition that would be expected to favour rather than impede glycogen resynthesis.

The time-course of glycogen resynthesis occurs in two distinct phases: an early (0-60 min) “insulin independent” phase where the rates of glycogen accumulation are high, followed by a late (1-48 h) “insulin dependent” phase when the rate of glycogen resynthesis is somewhat slower (19, 30). Somewhat surprisingly, most studies that have employed a carbohydrate-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 carbohydrate was ingested. In that study, resynthesis rates following caffeine plus carbohydrate ingestion were 72 mmol·kg⁻¹ d.w.h⁻¹ for the 30 min of recovery then declined during the subsequent 90 min and remained constant during the remainder of the 5 h recovery period such that the overall, the net rate of resynthesis was ~50 mmol·kg⁻¹ d.w.h⁻¹. In contrast to these findings, our results reveal that rates of muscle glycogen synthesis were similar (~60 mmol·kg⁻¹ d.w.h⁻¹) after 1 h of recovery for both treatments, but remained elevated with the co-ingestion of caffeine and carbohydrate throughout the 4 h observation period. Of note was 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 co-ingestion
of caffeine with carbohydrate (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 current investigation, Battram et al. (1) using a similar exercise regimen and caffeine ingestion protocol have previously reported elevations in serum FFA levels throughout 5 hr of recovery. In that study (Battram et al. 2004), the caffeine-induced increases in FFA concentration declined as insulin levels increased in response to frequent carbohydrate 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 Ca²⁺ 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 membrane (10). In an effort to identify a possible mechanism for the greater rates of muscle glycogen accumulation after the co-ingestion of caffeine with carbohydrate during recovery, we measured several signalling proteins considered to play putative roles in glucose transport into skeletal muscle. We observed that phosphorylation of CAMK¹¹°²⁸⁶ was similar between trials immediately post-exercise and also following 1 h of recovery, the latter being the time period during which rates of glycogen resynthesis were also the same (Figure 2A). However, at the end of the 4 hr
recovery period CAMK$_{\text{Thr}286}$ phosphorylation was significantly higher when caffeine was coingested with carbohydrate than when carbohydrate was ingested alone (Figure 2A). While the recovery period from 2-4 hr in the caffeine trial coincided with greater rates of glycogen resynthesis (and thus, presumably glucose transport into muscle), without additional biopsies we cannot determine if this outcome was associated with increased signalling to glucose transport through CAMK.

We observed a reduction in the exercise-induced phosphorylation of AMPK$_{\text{Thr172}}$ after 1 h of recovery that was similar for both ingestion protocols (Figure 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 co-ingestion of caffeine with carbohydrate, AMPK phosphorylation remained similar between treatments (Figure 2B). While our results do not support a role for AMPK in caffeine-induced glucose transport, Jensen et al. (20) have 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$\alpha$-1 activity and ACC$\beta$ 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$^{2+}$ signalling (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 current 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 signalling cascades to mechanisms important for GLUT4 translocation (for review see (24)). Akt is a serine/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 signalling to glucose transport in the working muscle. Glycogen synthase kinase-3 (GSK3) is a physiological target of Akt: phosphorylation of GSK3 decreases its activity towards glycogen synthase, which leads to increased glycogen synthesis (9). Isoform-specific silencing of Akt isoforms reveal 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\(^{\text{Ser473}}\) with the cessation of exercise for both treatment conditions (Figure 2C). The increase tended to be higher after the ingestion of caffeine with carbohydrate 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 and have implicated a role in glucose uptake. However, further work will be needed to test the hypothesis that caffeine induces an increase in Akt signalling 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 carbohydrate has an additive effect on rates on post-exercise muscle glycogen accumulation. Part of this effect may be due to the higher blood glucose and insulin concentrations observed after the co-ingestion of carbohydrate with caffeine, compared to when carbohydrate was ingested alone. The overall (4 h) rate of resynthesis observed in the present investigation (~60 mmol.kg\(^{-1}\) d.w.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 post-exercise glycogen resynthesis rates to the same extent remains to be determined.
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REFERENCES


FIGURE LEGENDS

Figure 1. Skeletal muscle glycogen content immediately post-exercise (0 h) and after 1 h and 4 h of recovery following cycling to volitional fatigue (~70% VO2 peak). During recovery subjects consumed 1 g carbohydrate kg\(^{-1}\) BM (CHO, open bars) or 1g carbohydrate kg\(^{-1}\) BM plus 8 mg kg\(^{-1}\) BM caffeine (CAFF, filled bars). * Significant difference vs. 0 h, # significant difference vs. 1 h, † significant difference CHO vs. caffeine 4 h (P < 0.05). All values are mean ± SD.

Figure 2. Skeletal muscle CAMK\(^{Thr286}\) (panel A), AMPK\(^{Thr172}\) (panel B) and Akt\(^{ser473}\) (panel C) phosphorylation immediately post-exercise (0 h) and after 1 h and 4 h of recovery following cycling to volitional fatigue (~70% VO2 peak). During recovery subjects consumed 1 g carbohydrate kg\(^{-1}\) BM (CHO, open bars) or 1g carbohydrate kg\(^{-1}\) BM plus 8 mg kg\(^{-1}\) BM caffeine (CAFF, filled bars). * Significant difference vs. 0 h, # significant difference vs. 1 h, † significant difference CHO vs. caffeine 4 h (P < 0.05). All values are mean ± SD.
Table 1. Blood glucose and plasma insulin concentrations at rest and following exercise (mean ± SD).

<table>
<thead>
<tr>
<th></th>
<th>Rest</th>
<th>0 h</th>
<th>0.5 h</th>
<th>1 h</th>
<th>1.5 h</th>
<th>2 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>CHO</td>
<td>3.9 ± 0.2</td>
<td>3.5 ± 0.3</td>
<td>6.1 ± 0.8*^</td>
<td>5.9 ± 0.8*^</td>
<td>5.4 ± 1.1*^</td>
</tr>
<tr>
<td></td>
<td>CAFF</td>
<td>4.0 ± 0.3</td>
<td>3.6 ± 0.6</td>
<td>6.1 ± 0.5*^</td>
<td>6.3 ± 0.8*^</td>
<td>6.0 ± 1.1*^†</td>
</tr>
<tr>
<td>Insulin</td>
<td>CHO</td>
<td>8.3 ± 3.2</td>
<td>4.3 ± 1.7</td>
<td>24.4 ± 11.7</td>
<td>24.5± 9.3</td>
<td>35.0 ± 10.9*^</td>
</tr>
<tr>
<td></td>
<td>CAFF</td>
<td>9.4 ± 3.3</td>
<td>4.2 ± 1.9</td>
<td>30.2 ± 15.2^</td>
<td>29.2 ± 7.8^</td>
<td>46.3 ± 16.7*^</td>
</tr>
</tbody>
</table>

During recovery subjects consumed 1 g carbohydrate ·kg⁻¹ BM (CHO) or 1g carbohydrate ·kg⁻¹ BM plus 8 mg·kg⁻¹ BM caffeine (CAFF). Glucose significantly different (P < 0.05) * vs. rest, ^ vs. 0 h, § vs. 0.5 h, ‡ vs. 1 h, # vs. 2 h, † CAFF vs. CHO. Insulin significantly different (P< 0.05) * vs. rest ^ vs. 0 h, § vs. all. Values are mean ±SD.
Table 2. Post-exercise muscle metabolite concentrations (mean ± SD).

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>CHO</th>
<th>0 h</th>
<th>1 h</th>
<th>4 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP (mmol/kg·d.w.)</td>
<td>25.4 ± 1.9</td>
<td>24.0 ± 2.1</td>
<td>26.1 ± 2.8</td>
<td></td>
</tr>
<tr>
<td>CAFF</td>
<td>25.4 ± 3.2</td>
<td>25.4 ± 2.4</td>
<td>24.2 ± 3.9</td>
<td></td>
</tr>
<tr>
<td>PCr (mmol/kg·d.w.)</td>
<td>72.1 ± 15.5</td>
<td>84.3 ± 11.9*</td>
<td>84.6 ± 8.9*</td>
<td></td>
</tr>
<tr>
<td>CAFF</td>
<td>73.7 ± 11.8</td>
<td>85.4 ± 10.8*</td>
<td>84.0 ± 12.7*</td>
<td></td>
</tr>
<tr>
<td>Free AMP (µmol/kg·d.w.)</td>
<td>0.82 ± 0.45</td>
<td>0.35 ± 0.22*</td>
<td>0.36 ± 0.13*</td>
<td></td>
</tr>
<tr>
<td>CAFF</td>
<td>0.69 ± 0.37</td>
<td>0.37 ± 0.18</td>
<td>0.34 ± 0.14*</td>
<td></td>
</tr>
</tbody>
</table>

During recovery subjects consumed 1 g carbohydrate ·kg⁻¹ BM (CHO) or 1 g carbohydrate ·kg⁻¹ BM plus 8 mg·kg⁻¹ BM caffeine (CAFF).

Significantly different (P< 0.05) * vs. 0 h within treatment. Values are mean ±SD.
Figure 1. Skeletal muscle glycogen content immediately post-exercise (0 h) and after 1 h and 4 h of recovery following cycling to volitional fatigue (~70% VO$_2$ peak). During recovery subjects consumed 1 g carbohydrate ·kg$^{-1}$ BM (CHO, open bars) or 1g carbohydrate ·kg$^{-1}$ BM plus 8 mg·kg$^{-1}$ BM caffeine (CAFF, filled bars). * Significant difference vs. 0 h, # significant difference vs. 1 h, † significant difference CHO vs. caffeine 4 h (P < 0.05). All values are mean ± SD.
Figure 2. Skeletal muscle CAMK<sub>Thr286</sub> (panel A), AMPK<sub>Thr172</sub> (panel B) and Akt<sub>Ser473</sub> (panel C) phosphorylation immediately post-exercise (0 h) and after 1 h and 4 h of recovery following cycling to volitional fatigue (~70% VO<sub>2</sub> peak). During recovery subjects consumed 1 g carbohydrate ·kg<sup>-1</sup> BM (CHO, open bars) or 1 g carbohydrate ·kg<sup>-1</sup> BM plus 8 mg·kg<sup>-1</sup> BM caffeine (CAFF, filled bars). * Significant difference vs. 0 h, # significant difference vs. 1 h, † significant difference CHO vs. caffeine 4 h (P < 0.05). All values are mean ± SD.