Impact of Caffeine and Protein on Postexercise Muscle Glycogen Synthesis

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ABSTRACT

Background: Both protein and caffeine co-ingestion with carbohydrate (CHO) have been suggested to represent effective dietary strategies to further accelerate post-exercise muscle glycogen synthesis in athletes. Purpose: To assess the impact of protein or caffeine co-ingestion on post-exercise muscle glycogen synthesis rates when optimal amounts of carbohydrate are ingested. Methods: 14 male cyclists were studied on 3 different test days. Each test day started with a glycogen depleting exercise session. This was followed by a 6 h recovery period, during which subjects received 1.2 g·kg⁻¹·h⁻¹ carbohydrate (CHO), or the same amount of carbohydrate with 0.3 g·kg⁻¹·h⁻¹ of a protein plus leucine mixture (CHO+PRO) or 1.7 mg·kg⁻¹·h⁻¹ caffeine (CHO+CAF). All drinks were enriched with [U⁻¹³C₆] labelled glucose to assess potential differences in the appearance rate of ingested glucose from the gut. Muscle biopsies were collected immediately after cessation of exercise and after 6 h of post-exercise recovery.

Results: The plasma insulin response was higher in CHO+PRO compared with CHO and CHO+CAF (P<0.01). Plasma glucose responses and glucose appearance rates did not differ between experiments. Muscle glycogen synthesis rates averaged 31±4, 34±4 and 31±4 mmol·kg dw⁻¹·h⁻¹ in CHO, CHO+PRO and CHO+CAF, respectively (P=NS). In accordance, histochemical analyses did not show any differences between net changes in type I and type II muscle fiber glycogen content between experiments. Conclusions: Co-ingestion of protein or caffeine does not further accelerate post-exercise muscle glycogen synthesis when ample amounts of carbohydrate (1.2 g·kg⁻¹·h⁻¹) are ingested.

Key words: insulin, leucine, glycogen synthase, amino acids, ¹³C-glucose.
**Introduction**

*Paragraph 1*

From a quantitative point of view, muscle glycogen represents the most important fuel source during moderate- to high-intensity endurance type exercise (37, 45). As a direct relationship exists between fatigue and muscle glycogen depletion (6, 7), post-exercise recovery time is mainly determined by the rate of muscle glycogen repletion. Ingestion of 1.2 g·kg\(^{-1}\)·h\(^{-1}\) carbohydrate, when provided at frequent intervals (i.e. every 15-30 min), allows optimal post-exercise muscle glycogen synthesis rates (8, 12, 19, 21, 23, 41, 44, 47). Further increasing carbohydrate intake, up to 1.6 g·kg\(^{-1}\)·h\(^{-1}\), does not augment post-exercise muscle glycogen storage (19). However, two nutritional intervention strategies have recently been suggested to accelerate muscle glycogen synthesis during post-exercise recovery.

*Paragraph 2*

Post-exercise amino acid and/or protein co-ingestion with carbohydrate has been established as an effective dietary strategy to strongly augment postprandial insulin release (35, 46, 48). As insulin stimulates both glucose uptake and glycogen synthase activity in skeletal muscle tissue (10, 22, 50), it has been suggested that co-ingestion of an insulinotropic amino acid and/or protein mixture can further accelerate post-exercise muscle glycogen synthesis. In accordance, we (47) as well as others (4, 20, 52) showed that amino acid and/or protein co-ingestion with carbohydrate (0.5-0.8 g·kg\(^{-1}\)·h\(^{-1}\)) accelerates post-exercise glycogen synthesis. However, several other studies reported no additional benefits of protein co-ingestion on post-exercise muscle glycogen synthesis when more than 1.0 g·kg\(^{-1}\)·h\(^{-1}\) carbohydrate was administered (19, 23, 44). It has been suggested that the latter might be attributed to the fact that muscle glycogen repletion
was assessed over a restricted timeframe of only 3 (23), and 4 h (19, 44) of post-exercise recovery. Such a short timeline might be insufficient to assess the full potential of insulin on post-exercise muscle glycogen synthesis rates, since glucose uptake and glycogen synthesis become more insulin dependent throughout the latter stages of the recovery period (22, 34). So far, no study has assessed the impact of co-ingesting an insulinotropic amino acid/protein mixture on post-exercise muscle glycogen synthesis during 6 h of recovery when an optimal amount of carbohydrate (1.2 g·kg⁻¹·h⁻¹) is provided.

**Paragraph 3**

Recently, Pedersen et al. (31) reported a substantial increase in post-exercise muscle glycogen synthesis rate when caffeine (2 mg·kg⁻¹·h⁻¹) was co-ingested with carbohydrate (1.0 g·kg⁻¹·h⁻¹). The latter was remarkable as previous work generally reports a negative effect of caffeine administration on glucose disposal (1, 17, 29, 33, 43). Though the mechanism(s) that might explain the proposed stimulating effect of caffeine co-ingestion on muscle glycogen repletion remained unresolved, the authors (31) speculated that caffeine co-ingestion might stimulate intestinal glucose absorption (49, 51). Studies are warranted to assess whether caffeine co-ingestion accelerates post-exercise muscle glycogen repletion when an optimal amount of carbohydrate (1.2 g·kg⁻¹·h⁻¹) is provided.

**Paragraph 4**

In the present study, we tested both hypotheses that co-ingestion of an insulinotropic amino acid/protein mixture or caffeine with 1.2 g carbohydrate·kg⁻¹·h⁻¹ accelerates post-exercise muscle glycogen synthesis when compared with ingestion of carbohydrate only. Therefore, we subjected
14 male cyclists to glycogen depletion exercise on 3 occasions, after which they ingested carbohydrate (1.2 g·kg⁻¹·h⁻¹), carbohydrate (1.2 g·kg⁻¹·h⁻¹) with an amino acid/protein mixture (0.3 g·kg⁻¹·h⁻¹), or carbohydrate (1.2 g·kg⁻¹·h⁻¹) with added caffeine (1.7 mg·kg⁻¹·h⁻¹) during a 6 h recovery period. Muscle biopsies were collected immediately after cessation of exercise and after 6 h of post-exercise recovery to assess the increase in muscle glycogen content. Furthermore, [U-¹³C₆] labelled glucose was administered orally to assess potential differences in the appearance rate of the ingested glucose in the circulation.

Methods

Paragraph 5

Subjects

Fourteen well-trained male cyclists participated in this study (age: 24±1 y, bodyweight: 71.6±2.5 kg, body mass index: 21.7±0.4 kg·m⁻², maximal workload capacity (Wₘₐₓ): 387±11 W, maximal oxygen uptake capacity (VO₂ₘₐₓ): 61.5±1.2 ml·kg⁻¹·min⁻¹). Subjects cycled at least 100 km per wk and had a training history of more than 3 y. Subjects were fully informed on the nature and possible risks of the experimental procedures, before their written informed consent was obtained. The study was approved by the Medical Ethical Committee of the MUMC+, Maastricht, the Netherlands.
Paragraph 6

Pretesting

All subjects participated in a screening session, which was performed one week prior to the first experiment. Subjects performed an incremental exhaustive exercise test (27) on an electronically braked cycle ergometer (Lode Excalibur, Groningen, The Netherlands) to assess \( \text{VO}_{2\text{max}} \) and \( \text{W}_{\text{max}} \).

Paragraph 7

Diet and activity prior the experiments

All subjects received the same standardized dinner (81±3 kJ·kg bw\(^{-1} \)) consisting of 58 energy% (En%) carbohydrate, 31 En% fat, and 11 En% protein) the evening prior to each test day. All volunteers refrained from any sort of exhaustive physical labour and/or exercise and kept their diet as constant as possible 2 days prior to each experimental day. In addition, subjects filled in food intake and physical activity questionnaires for 2 days prior to the start of the first experiment, which were used to standardize food intake and physical activity prior to the second and third experimental day. Furthermore, subjects were instructed to refrain from caffeine containing food products like coffee, tea, cola and chocolate, for 2 days prior to each experimental day. Subjects’ regular caffeine use was 3.5±0.6 units a day, in which a unit represents one cup of coffee with a caffeine content of ~70 mg.
**Paragraph 8**

**Design**

Subjects performed 3 randomized tests, each separated by at least one week. During each test they were first subjected to a glycogen depletion protocol. Thereafter, subjects were studied for 6 h while ingesting only carbohydrate in the control trial (CHO), carbohydrate plus a protein plus leucine mixture in the CHO+PRO trial or carbohydrate plus caffeine in the CHO+CAF trial. During the 6 h post-exercise recovery period subjects remained in supine rest. Beverages were provided every 30 min and tests were performed in a randomized, double-blind order. Muscle biopsies were taken immediately after exercise and at the end of the 6 h recovery period to assess changes in muscle glycogen content.

**Paragraph 9**

**Experimental protocol**

Subjects reported to the laboratory at 8.00 a.m. following an overnight fast. Muscle glycogen depletion was established by performing an intense exercise protocol on a cycle ergometer (26). This muscle glycogen depletion protocol started with a 10 min warm-up period at a 50% $W_{\text{max}}$ workload. Thereafter, subjects were instructed to cycle 2-min block periods at alternating workloads of respectively 90 and 50% $W_{\text{max}}$. This was continued until subjects were no longer able to complete the 2 min at 90% $W_{\text{max}}$. That moment was defined as the inability to maintain cycling speed at 60 revolutions per min. At that moment the high intensity block was reduced to 80% $W_{\text{max}}$. Again subjects had to cycle until they were unable to complete a 2-min block at 80% $W_{\text{max}}$, after which the high intensity block was reduced to 70% $W_{\text{max}}$. Subjects were allowed to stop when pedaling speed could not be maintained at 70% $W_{\text{max}}$. Water was provided ad libitum.
during the exercise protocol of the first test day, and the same amount of water was provided during the second and third test day. A fan was placed 1 m from the subjects to provide cooling and air circulation during the exercise protocol. After cessation of exercise a muscle biopsy was taken from the *vastus lateralis* muscle. Thereafter, a Teflon catheter (Baxter BV, Utrecht, the Netherlands) was inserted in an antecubital vein, a resting blood sample was taken and subjects received the first bolus of the test drink (*t*=0 min). Subjects were observed for the following 6 h during which they received a beverage with a volume of 3 ml·kg⁻¹ every 30 min until *t*=330 min. Blood samples were taken at 15 min intervals for the first 90 min of recovery and every 30 min thereafter until *t*=360 min. Immediately after acquiring the final blood sample, a second biopsy was taken from the *vastus lateralis* muscle of the other leg.

*Paragraph 10*

**Questionnaires**

Subjects were asked to fill out a questionnaire using a 10-point scale (1 = not at all, 10 = very, very much) at *t* = -5, 175 and 355 min. This questionnaire contained questions regarding the presence of gastrointestinal (GI) distress and addressed the following complaints: nausea, bloated feeling, belching, stomach problems and GI cramping, vomiting, diarrhoea, the urge to urinate and/or defecate, headache, and dizziness. One question regarding the taste of the test drink was also conducted (1 = horrible, 10 = very tasty).
Paragraph 11

**Beverages**

Subjects received a beverage volume of 3 ml·kg⁻¹ every 30 min during recovery, to ensure a given dose of 1.2 g·kg⁻¹·h⁻¹ carbohydrate (CHO), 1.2 g·kg⁻¹·h⁻¹ carbohydrate with 0.2 g·kg⁻¹·h⁻¹ protein hydrolysate and 0.1 g·kg⁻¹·h⁻¹ leucine (CHO+PRO), or 1.2 g·kg⁻¹·h⁻¹ carbohydrate with 1.7 mg·kg⁻¹·h⁻¹ caffeine (CHO+CAF). To all test drinks 0.32 g·L⁻¹ [U-¹³C₆] labelled glucose was added. The carbohydrate source consisted of 50% glucose and 50% maltodextrin (AVEBE, Veendam, The Netherlands). The casein protein hydrolysate (PeptoPro®, 85.3% protein) was prepared by DSM Food Specialties (Delft, The Netherlands). Leucine was obtained from Ajinomoto (Tokyo, Japan), caffeine from Fagron (Nieuwekerk a/d IJssel, The Netherlands) and [U-¹³C₆] glucose from Cambridge Isotope Laboratories Inc. (Andover, U.S.A.). To make the taste comparable, all solutions were flavoured by adding 0.05 g·L⁻¹ sodium saccharinate, 0.9 g·L⁻¹ citric acid and 5.0 g·L⁻¹ cream vanilla flavour (Givaudan Nederland B.V., Barneveld, The Netherlands). Treatments were performed in a randomized order, with test drinks provided in a double-blind fashion.

Paragraph 12

**Muscle biopsies**

Muscle biopsies were obtained from the middle region of the *vastus lateralis* muscle (~15 cm above the patella) and approximately 2 cm below the entry through the fascia by means of the percutaneous needle biopsy technique described by Bergström *et al.* (5). All samples were carefully freed from any visible adipose tissue and blood, immediately frozen in liquid nitrogen.
(biochemistry) or in liquid nitrogen cooled isopentane (histochemistry), and stored at –80 °C until subsequent analysis.

Paragraph 13

Plasma analysis

Blood samples (8 ml) were collected in EDTA containing tubes and centrifuged at 1000g and 4°C for 10 min. Aliquots of plasma were frozen in liquid nitrogen and stored at –80°C until analysis. Plasma glucose (Uni Kit III, 07367204, Roche, Basel, Switzerland), lactate (18) and FFA (NEFA-C, Wako Chemicals, Neuss, Germany) concentrations were analyzed with a COBAS-FARA semiautomatic analyzer (Roche). Insulin was analyzed by radio immunoassay (Linco, Human Insulin RIA kit, LINCO Research Inc., St. Charles, MO, USA). Concentrations of plasma catecholamines (39), sampled with heparin and put into glutathione containing tubes on ice, and plasma caffeine (Clin Rep Komplettkit für Theophyllin, Theobromin und Coffein, Recipe Chemical+Instruments Labortechnik, Munich, Germany) were determined by using high-performance liquid chromatography (HPLC). Plasma [U-13C6] glucose enrichment was determined using the method of Pickert et al. (32), modified for use with gas chromatography-combustion-IRMS (GC-C-IRMS). Briefly, plasma samples were extracted with methanol-chloroform (2.3:1, v/v) and then chloroform-water (pH 2.0) (2:1, v/v). After drying under nitrogen gas, samples were derivatized to butylboronic acid acetate, using butylboronic acid as derivatizing agent. The glucose derivative (1 μL) was injected into the GC (split ratio 1:15) and analyzed by GC-C-IRMS (GC, Trace GC Ultra; C, GC Combustion III; IRMS, Delta Plus XP; all Thermo Finnigan, Herts, UK). The measured 13C enrichment was corrected by a factor of 16/6
to account for isotopic carbon dilution from the butylboronic acid-acetyl derivative. Plasma [U-
\textsuperscript{13}C\textsubscript{6}] glucose enrichments are expressed as tracer-to-tracee ratios.

**Paragraph 14**

**Muscle analyses**

*Biochemical analysis:* \(~25\) mg wet weight muscle tissue was freeze-dried after which collagen, blood and other non-muscle fiber material were removed from the muscle fibers under a microscope. The isolated muscle fiber mass (\(~5\) mg) was weighed and \(1000\ \mu\text{L}\) \(1\text{M HCl}\) was added. After heating for \(3\) h at \(100^\circ\text{C}\) to hydrolyze the glycogen to glycosyl units and cooling down to room temperature, \(400\ \mu\text{L}\) of the solution was neutralized by adding \(250\ \mu\text{l}\) Tris/ KOH (precise amount of Tris/KOH is determined by titration to pH \(7.0\)). Thereafter \(150\ \mu\text{L}\) of this solution was analyzed for glucose concentration (Uni Kit III, 07367204, La Roche, Basel, Switzerland) with a COBAS FARA semi automatic analyzer (Roche).

**Paragraph 15**

*Histochemical analysis:* **multiple** serial sections (5 \(\mu\text{m}\)) from biopsy samples collected immediately after \((t=0\ \text{min})\) and \(6\) h after exercise \((t=360\ \text{min})\) were thaw-mounted on uncoated, pre-cleaned glass slides for each subject. The antibody against laminin was used to select individual muscle fibers and the antibody against human myosin heavy chain I to differentiate between the type I and II muscle fibers. To assess intramyocellular glycogen content we used the modified PAS stain as described previously (38). After 24 h, glass slides were examined using a Nikon E800 fluorescence microscope (Uvikon, Bunnik, The Netherlands) coupled to a Basler A113 C progressive scan colour CCD camera, with a Bayer colour filter. Epifluorescence signal
was recorded using a (FITC) excitation filter (465–495 nm) for laminin, a 4’,6-diamidino-2-phenylindole (DAPI) UV excitation filter (340–380 nm) for nuclear staining and Texas red excitation filter (540-580 nm) for muscle fiber type. Digitally captured images were processed and analysed using Lucia 4.8 software (Nikon, Düsseldorf, Germany). PAS stained sections were captured in full colour using bright field light microscopy. The PAS signal was recorded for each muscle fiber, resulting in a total of 123±4 muscle fibers analysed for each muscle cross-section (85±3 type I and 38±2 type II muscle fibers). The bright-field images of the PAS stains were converted post hoc to 8 bit greyscale values. The mean optical density of the PAS-raised signal per individual fiber was determined by averaging the optical density measured in every pixel in the cell, corrected for the mean optical density of the background stain measured in a field-of-view containing no muscle fibers. Mixed muscle glycogen content, as determined by PAS staining, has previously been shown to correlate well with muscle glycogen content measured using the biochemical assay (38).

**Paragraph 16**

**Statistics**

All data are expressed as means±SEM. The plasma insulin and glucose responses were calculated as area under the curve. A 2-factor repeated measures analysis of variance (ANOVA) with time and treatment as factors was used to compare differences between treatments over time. In case of significant F-ratio’s, Bonferroni post-hoc tests were applied to locate the differences. For non-time dependent variables, a paired Student’s t-test was used to compare differences between treatment and control. The results from the questionnaires were analysed by
the Friedman non-parametric test. Statistical significance was set at P<0.05. All calculations were performed using SPSS Statistics 15.0 (SPSS Inc., Chicago, USA).

**Results**

**Paragraph 17**

**Glycogen depletion protocol**

Maximal workload capacity measured during the pretesting averaged 387±11 W (5.4±0.1 W kg⁻¹). Consequently, average workload settings in the depletion protocol were 194±5, 271±8, 310±9 and 349±10 W at 50, 70, 80 and 90% W_max, respectively. On average subjects cycled a total of 23±3, 23±2, and 23±2 high-intensity blocks, which resulted in a total cycling time of 106±10, 104±10, and 106±10 min in the CHO, CHO+PRO and CHO+CAF experiment, respectively. Total cycling time did not differ between experiments.

**Paragraph 18**

**Drink ingestion and gastrointestinal complaints**

All drinks were well-tolerated by the subjects, though some subjects had difficulty ingesting the last 2 test drinks. Nonetheless, 95±2 % of the total volume of test drink (2.6±0.9 L) was ingested, with no differences between treatments. The main complaints according to the questionnaires were bloated feeling, belching, and the urge to urinate. There were no differences between experiments, although the taste of the CHO+PRO drink was rated significantly lower than the CHO and CHO+CAF drinks (2.5±0.2, 5.1±0.3 and 4.5±0.4, respectively; P<0.01).
Paragraph 19

**Plasma analyses**

In all experiments, plasma insulin concentrations increased during the first 90 min of post-exercise recovery, after which concentrations plateaued (Figure 1A). Plasma insulin concentrations were higher in the CHO+PRO compared with CHO treatment (P<0.05; Figure 1B). Plasma glucose concentrations increased during the first 60 min of post-exercise recovery, after which concentrations declined to baseline levels (Figure 2A). Plasma glucose responses did not differ between experiments (Figure 2B). Plasma [U-13C6] glucose enrichments are shown in Figure 3. No differences in plasma [U-13C6] glucose enrichments were observed between treatments.

Paragraph 20

Plasma lactate, free fatty acids (FFA), adrenalin, and noradrenalin concentrations are presented in Table 1. Plasma lactate concentrations over time were lower in CHO+PRO compared with CHO (P=0.012). There were no differences between CHO and CHO+CAF. Plasma FFA concentrations over time were higher in CHO+CAF compared with CHO (P<0.01). There were no differences between CHO and CHO+PRO. Plasma adrenalin concentrations over time did not differ between experiments. Plasma noradrenalin concentrations over time were lower in CHO+PRO compared with CHO. There were no differences in plasma noradrenalin concentrations over time between the CHO and CHO+CAF experiment.
Paragraph 21

Plasma caffeine concentrations increased to 4.7±0.2 and 9.3±0.5 mg L⁻¹ or 24.3±1.1 and 47.9±2.4 μmol L⁻¹ at t=180 and t=360 min., respectively (P<0.01) in the CHO+CAF experiment. Caffeine concentrations were below the detection limit (<0.05 mg L⁻¹) in the CHO and CHO+PRO experiment.

Paragraph 22

Muscle analyses

Post-exercise muscle glycogen concentrations did not differ between experiments and averaged 172±40, 184±32 and 138±22 mmol·kg⁻¹·dw⁻¹ in the CHO, CHO+PRO and CHO+CAF treatment, respectively. After 6 h of post-exercise recovery, muscle glycogen concentrations had increased to 360±31, 384±25 and 326±24 mmol·kg⁻¹·dw⁻¹, respectively (NS). Glycogen resynthesis rates are shown in Figure 4. Histochemical analyses of the muscle biopsies revealed no differences in muscle glycogen content between experiments or between type I and type II fibers (Figure 5). There was a strong significant correlation between mixed muscle glycogen content, as determined by PAS staining, and muscle glycogen content measured using the biochemical assay, with a Pearson correlation coefficient of 0.84 (P<0.01).

Discussion

Paragraph 23

The present study shows that co-ingesting 0.3 g·kg⁻¹·h⁻¹ of an insulinogetic protein plus leucine mixture or 1.7 mg·kg⁻¹·h⁻¹ caffeine with 1.2 g·kg⁻¹·h⁻¹ carbohydrate does not further accelerate
post-exercise muscle glycogen synthesis when compared with the ingestion of 1.2 g·kg⁻¹·h⁻¹ carbohydrate only.

*Paragraph 24*

The rate of post-exercise muscle glycogen synthesis depends on numerous factors, which include the magnitude of muscle glycogen depletion, the amount of carbohydrate ingestion, the rate of gastric emptying and intestinal glucose uptake, and the insulin stimulated uptake of glucose and subsequent conversion to muscle glycogen by glycogen synthase (3, 22).

*Paragraph 25*

The exercise protocol in our study resulted in post-exercise muscle glycogen concentrations that did not differ between treatments (i.e. 172±40, 184±32 and 138±22 mmol·kg dw⁻¹ in the CHO, CHO+PRO and CHO+CAF treatment, respectively). The post-exercise muscle glycogen levels are comparable to values published previously applying similar exercise protocols to strongly reduce muscle glycogen levels (23, 47). Muscle glycogen synthesis has been shown to occur in two distinct phases (15, 28, 34, 36), with the first insulin-independent phase being characterized by higher muscle glycogen synthesis rates. Several studies have shown that this fast phase of muscle glycogen synthesis occurs when concentrations are reduced to less than 150 mmol·kg dry weight⁻¹ (22, 28, 30, 34). In our study, post-exercise muscle glycogen concentrations were below this level in the CHO+CAF experiment only, but this did not result in higher muscle glycogen synthesis rates over the 6 h recovery phase.
Paragraph 26

Co-ingestion of protein, protein hydrolysates, and/or free amino acids with carbohydrate have been shown to strongly augment postprandial insulin release (24, 35, 46, 48). In the present study, we confirm the insulinotropic potential of protein and free leucine co-ingestion, with a 76±11% greater insulin response in the CHO+PRO versus CHO experiment. However, the greater insulin response did not augment post-exercise muscle glycogen synthesis. The latter seems to be in agreement with three other studies (19, 23, 44), that report no additional benefits of protein co-ingestion on post-exercise muscle glycogen synthesis when large amounts of carbohydrate were administered during relatively short recovery periods of 3 and 4 h. Since glucose uptake and glycogen synthesis become more insulin dependent throughout the latter stages of post-exercise recovery (22, 34), we hypothesized that the glycogen stimulating effect of protein co-ingestion with an optimal amount of carbohydrate would become apparent following a more extended recovery period of 6 h. In contrast to our hypothesis, data from the present study indicate that circulating insulin levels are not the rate limiting factor for muscle glycogen synthesis when post-exercise carbohydrate intake is optimal (1.2 g·kg⁻¹·h⁻¹).

Paragraph 27

From the present findings, we conclude that a further elevation of circulation insulin levels does not accelerate muscle glycogen synthesis during post-exercise recovery. Nonetheless, it could be speculated that stimulating insulin release could still be beneficial for other aspects of recovery. While recent studies have shown that increasing insulin does not further stimulate muscle protein synthesis when ample protein is ingested (16, 25, 40), it may inhibit exercise induced muscle protein breakdown. The latter might improve net protein balance and, as such, improve post-

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exercise muscle reconditioning. Furthermore, elevating post-exercise insulin levels might accelerate the replenishment of liver glycogen stores, which is an aspect of post-exercise recovery that has received much less attention (11, 14).

*Paragraph 28*

It might be speculated that the increased osmolality and energy density of the CHO+PRO beverage delayed gastric emptying, thereby decreasing the delivery of exogenous carbohydrate for muscle glycogen synthesis. However, in the present study, the co-ingestion of protein or caffeine did not result in differences in exogenous [U-13C6] glucose appearance, suggesting that gastric emptying rates did not differ between treatments.

*Paragraph 29*

Recently, Pedersen et al. (31) showed a 66% greater increase in muscle glycogen synthesis rate during post-exercise recovery when caffeine was co-ingested with carbohydrate. The latter seems at odds with previous work in which no stimulating effect of caffeine supplementation on muscle glycogen synthesis has been observed (2). However, in the latter study subjects were administered caffeine prior to and during exercise, while the carbohydrate containing beverages were provided during post-exercise recovery. Consequently, it could be suggested that caffeine can only exert its effect on muscle glycogen synthesis when co-ingested with the carbohydrate supplements. The mechanism by which caffeine co-ingestion might accelerate post-exercise muscle glycogen synthesis remains unclear. In resting conditions, caffeine ingestion has been shown to actually impair insulin-mediated glucose disposal (1, 17, 29, 33, 43), likely attributed to β-adrenergic stimulation and/or adenosine receptor antagonism (1, 43). However, exercise
seems to attenuate these detrimental effects of caffeine on insulin action in skeletal muscle tissue (42). Furthermore, studies that were performed during exercise suggest that caffeine co-ingestion with carbohydrate can stimulate intestinal glucose absorption by stimulating the jejunal sodium-glucose transporter protein SGLT1 via cAMP (49, 51).

**Paragraph 30**

To assess the potential stimulating properties of caffeine co-ingestion on the intestinal uptake of ingested glucose during post-exercise recovery, we added a [U-13C6] labelled glucose tracer to the beverages. The increase in plasma [U-13C6] glucose enrichments did not differ between treatments, implying that there were no differences in exogenous glucose appearance rates between treatments. Consequently, our findings do not support the suggestion that caffeine co-ingestion stimulates intestinal glucose absorption during post-exercise recovery. In accordance, caffeine co-ingestion did not accelerate muscle glycogen synthesis rates. There is no clear explanation for the differences between our findings and the recent work by Pedersen *et al.* (31). The apparent discrepancy may be attributed to differences in study design. In the present study we applied the exhaustive exercise session in the morning following an overnight fast, whereas Pedersen *et al.* included an additional exercise session the evening prior to the experimental day followed by the ingestion of a low-carbohydrate diet. Consequently, post-exercise muscle glycogen levels were generally lower in the study by Pedersen *et al.* when compared with the present findings. However, these differences would unlikely modulate the proposed efficacy of caffeine to accelerate intestinal glucose absorption or subsequent muscle glycogen synthesis. Furthermore, there were distinct differences in carbohydrate supplementation regimen between studies. Pedersen *et al.* provided their subjects with 1.0 g·kg⁻¹·h⁻¹ carbohydrate with/without 2.0
mg·kg⁻¹·h⁻¹ caffeine at 2 h intervals during a 4 h recovery period. In contrast, we provided subjects with 1.2 g·kg⁻¹·h⁻¹ carbohydrate with or without 1.7 mg·kg⁻¹·h⁻¹ caffeine via boluses provided every 30 min during a more extensive 6 h recovery period. A more frequent provision of carbohydrate has generally shown to maximize the rate of post-exercise glycogen repletion. Though the absolute amount of caffeine that was provided during the recovery phase was similar between studies, Pedersen et al. provided their supplements at 0 and 2 h of post-exercise recovery. The latter might explain the slightly higher plasma caffeine concentrations in their study. However, this is unlikely responsible for the ~70% greater glycogen resynthesis rate in the carbohydrate plus caffeine treatment. So far there is little evidence and/or mechanistic background to suggest that caffeine co-ingestion following exercise will accelerate post-exercise muscle glycogen synthesis when ample amounts of carbohydrate are provided at (more) frequent intervals.

**Paragraph 31**

After 6 h of post-exercise recovery, muscle glycogen concentrations had increased to 360±31, 384±25 and 326±24 mmol·kg dw⁻¹ in the CHO, CHO+PRO and CHO+CAF experiment, respectively. We did not measure basal muscle glycogen concentrations, but previous studies have reported pre-exercise values in athletes to range between 500-600 mmol·kg dry weight⁻¹ (9, 13). The latter implies that muscle glycogen stores can not entirely be replenished within 6 h of post-exercise recovery even when ingesting such large amounts of carbohydrate with or without additional protein or caffeine. Moreover, for athletes training or competing twice daily or recovering overnight it is not always feasible to ingest such substantial amounts of carbohydrate. It has previously been shown that the co-ingestion of protein with a smaller amount of
carbohydrate stimulates muscle glycogen synthesis to the same extend when compared to the ingestion of a greater amount of carbohydrate (47). Based on those findings, it was speculated that the greater post-prandial insulin response following protein co-ingestion facilitates the uptake of the ingested carbohydrate in the more insulin sensitive tissues, like skeletal muscle. Therefore, under circumstances where the total amount of carbohydrate that can be ingested is limited, it might be preferred to co-ingest some protein to facilitate glucose uptake in muscle. However, more work is warranted to determine the impact of a greater post-prandial insulin response on glucose uptake and glycogen deposition in various tissues. Furthermore, the co-ingestion of caffeine during short-term recovery might enhance subsequent exercise performance via its stimulating effect on neuromuscular function.

**Paragraph 32**

In summary, we show that co-ingestion of 0.3 g·kg⁻¹·h⁻¹ protein with 1.2 g·kg⁻¹·h⁻¹ carbohydrate during post-exercise recovery strongly increases insulin release, but does not further accelerate muscle glycogen synthesis. Furthermore, co-ingestion of 1.7 mg·kg⁻¹·h⁻¹ caffeine with 1.2 g·kg⁻¹·h⁻¹ carbohydrate has no impact on the uptake of ingested glucose from the gut and does not increase post-exercise muscle glycogen synthesis. We conclude that co-ingestion of an insulinotropic amino acid/protein mixture or caffeine does not further accelerate post-exercise muscle glycogen synthesis when an optimal amount of carbohydrate (1.2 g/kg/h) is already ingested.
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MB and LJCvL designed the study. MB organized and carried out the clinical experiments. JvK and JMS performed the stable isotope tracer analyses. MB performed the statistical analysis and wrote the manuscript together with HK and LJCvL.

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References


Figure legends

Figure 1. Mean (±SEM) plasma insulin concentrations over time (A) and total insulin responses, measured as area under the curve (B), in the CHO, CHO+PRO and CHO+CAF experiment. Data were analyzed with ANOVA repeated measures (treatment x time). *: significantly different from CHO (P<0.05).

Figure 2. Mean (±SEM) plasma glucose concentrations over time (A) and total glucose responses, measured as area under the curve (B), in the CHO, CHO+PRO and CHO+CAF experiments. Data were analyzed with ANOVA repeated measures (treatment x time). No differences were observed between experiments.

Figure 3. Mean (±SEM) plasma [U-13C6] glucose enrichments over time in the CHO, CHO+PRO and CHO+CAF experiments. Data were analyzed with ANOVA repeated measures (treatment x time). No differences were observed between experiments.

Figure 4. Mean (±SEM) muscle glycogen synthesis rates (mmol·kg dw⁻¹·h⁻¹) in the CHO, CHO+PRO and CHO+CAF experiments. Data were analyzed with ANOVA repeated measures (treatment x time). No differences were observed between experiments.

Figure 5. Mean (±SEM) muscle glycogen concentration in type I and type II muscle fibres prior to (pre) and after (post) 6 h of post-exercise recovery in the CHO, CHO+PRO and CHO+CAF experiments. Data were analyzed with ANOVA repeated measures (treatment x time). *: significantly different from values immediately after cessation of exercise (P<0.05).
Figure 1

A. Graph showing plasma insulin concentration (mU·L⁻¹) over time (min) for CHO, CHO+PRO, and CHO+CAF conditions. 

B. Bar graph comparing plasma insulin response (mU·L⁻¹·6h⁻¹) across CHO, CHO+PRO, and CHO+CAF conditions.
Figure 2

A: Plasma glucose concentration (mmol L⁻¹) over time (min) for CHO, CHO+PRO, and CHO+CAF conditions.

B: Plasma glucose response (mmol L⁻¹·6h⁻¹) for CHO, CHO+PRO, and CHO+CAF conditions.
Figure 5

[Graph showing muscle glycogen concentration (AU) with bars for Pre and Post conditions under CHO, CHO+PRO, and CHO+CAF treatments, comparing Type I and Type II fibres.]
### Table 1  Plasma lactate, FFA, adrenalin and noradrenalin concentrations

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<th>Time</th>
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<th>Experiment CHO+PRO</th>
<th>Experiment CHO+CAF</th>
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Values are expressed as mean±SEM. Data were analysed with ANOVA repeated measures (treatment x time). * Significantly different from CHO.