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Co-ingestion of protein and carbohydrate in the early recovery phase improves endurance performance despite like glycogen degradation and AMPK phosphorylation

Co-ingestion of protein and carbohydrate in the early recovery phase improves endurance performance despite like glycogen degradation and AMPK phosphorylation

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Running title: Protein intake stimulates recovery after exhaustive exercise

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ABSTRACT

The present study compared the effects of post exercise carbohydrate plus protein (CHO+PROT) and carbohydrate (CHO) only supplementation on muscle glycogen metabolism, anabolic cell signalling and subsequent exercise performance. Nine endurance-trained males cycled twice to exhaustion (muscle glycogen decreased from ~495 to ~125 mmol·kg dw^{-1}) and received either CHO only (1.2 $g \cdot kg^{-1} \cdot h^{-1}$) or CHO+PROT (0.8/0.4 $g \cdot kg^{-1} \cdot h^{-1}$) during the first 90 min of recovery. Glycogen content was similar before the performance test after 5 h of recovery. Glycogen synthase (GS) fractional activity increased after exhaustive exercise and remained activated 5 h after despite substantial glycogen synthesis (176.1±19.1 and 204.6±27.0 mmol·kg dw^{-1} in CHO and CHO+PROT, respectively; p=0.15). Phosphorylation of GS at site 3 and site 2+2a remained low during recovery. After the 5 h recovery, cycling time to exhaustion was improved by CHO+PROT supplementation compared to CHO supplementation (54.6±11.0 vs 46.1±9.8 min; p=0.009). After the performance test, muscle glycogen was equally reduced in PRO+CHO and CHO. Akt Ser^{473} and p70s6k Thr^{389} phosphorylation was elevated after 5 h of recovery. There were no differences in Akt Ser^{473}, p70s6k Thr^{389} or TSC2 Thr^{1462} phosphorylation between treatments. Nitrogen balance was positive in CHO+PROT (19.6±7.6 mg nitrogen·kg^{-1}, p=0.04) and higher than CHO (-10.7±6.3 mg nitrogen·kg^{-1}, p=0.009). **Conclusion:** CHO+PROT supplementation during exercise recovery improved subsequent endurance performance relative to consuming CHO only. This improved performance after CHO+PROT supplementation could not be accounted for by differences in glycogen metabolism or anabolic cell signaling, but may have been related to differences in nitrogen balance.

Keywords: Exercise, glycogen synthase, Akt/PKB, protein synthesis, nitrogen balance

New & Noteworthy

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

INTRODUCTION

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

Co-ingestion of protein and carbohydrate has been reported to increase the rate of glycogen synthesis compared to carbohydrate only (5; 70). The mechanisms contributing to recovery of performance after endurance exercise include glycogen synthesis (23). However, protein intake after exercise stimulates protein synthesis (15; 21; 37; 65), limits muscle damage (9), modulates transcription (51) and increases activation of anabolic signalling pathways (12; 49). Some studies have not found that co-ingestion of protein and carbohydrate recovers performance better than carbohydrate only (5; 43; 47; 52; 53). The reasons that protein intake in the recovery period did not improve performance in these studies may be due to the type and duration of exercise before the dietary interventions, the length of recovery period, nitrogen balance and the test used to evaluate performance. Importantly, it is necessary to develop protocols where co-ingestion of protein and carbohydrate consistently improves recovery of performance better than intake of carbohydrate only. Without such protocols, it is impossible to illuminate the mechanisms responsible for the improved performance after intake of protein.

Muscle biopsies have been taken in only one study where co-ingestion of protein and carbohydrate improved recovery of endurance performance relative to carbohydrate alone (12). Ferguson-Stegall et al. showed that glycogen synthesis was similar but activation of anabolic signalling was increased with co-ingestion of protein and carbohydrate (12). Many

other studies have reported improved activation of anabolic signalling after intake of protein (42; 46; 49; 51; 56; 65), but none of these studies investigated the effect of protein intake on performance.

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

MATERIALS AND METHODS

Nine males training for competition in triathlon (n=2) or cycling (Mountain biking; n=7) completed the study. Inclusion criteria were 1) bicycle training more than twice a week for the last six months, 2) $VO_{2max} \geq 50$ ml·kg^{-1}·min^{-1}, 3) age 18-40 years, and 4) no known diseases. Characteristics of the participants were age: 26.7±1.7 years; weight: 76.4±3.2 kg; height: 182.4±2.2 cm; maximal heart rate: 188.0±2.2 bpm and VO_{2max}: 58.1±1.7 ml·kg^{-1}·min^{-1}. The participants were informed individually about the study and biopsy procedures, and each participant signed an informed consent. The study was approved by the Regional Ethical Committee of Midtjylland, Denmark (J. No 1-10-72-23-13)) and conducted in accordance to the principles from the Declaration of Helsinki. The study was a double-blinded, cross-over design, with one week between the two interventions. Random assignment into groups was done by minimization (55) using publicly available software (Minim: Allocation by minimization in clinical trials).

Testing of VO_{2peak} and incremental test

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

Incremental test: On the first test day, participants performed 1) an incremental exercise test to establish the relationship between work rate and oxygen uptake, and 2) measure peak oxygen consumption (VO_{2peak}). Initially, there was a short (3-5 min) warm-up at 100 Watt during which participants selected a cadence between 90 and 100 revolutions per min (RPM). This self-selected RPM was used in all testing throughout the study. The incremental test then started at 125-175 Watt depending on training condition, and increased 25 Watt every 5th min. VO_2 was measured during the last 90 s of each load. After 4 min of cycling at each exercise work rate, a capillary blood sample (Accu-Check, Safe-T-Pro Plus; Manheim, Deutschland) was taken for measurement of blood lactate and glucose. Capillary blood glucose was measured with a HemoCue Glucose 201+ analyser (Angelholm, Sweden). For lactate analyses, a micro haematocrit tube (55 μl, Radiometer, Copenhagen, Denmark) was filled and 23 μl of blood was immediately pipetted into a YSI Analyser (Yellow Spring Instruments 1500 SPORT, Ohio, USA). The incremental test terminated when the blood lactate concentration was higher than 4 mM. The YSI Analyser was calibrated with a standard of 5 mM lactate each day. HR was measured continuously during all testing with a Polar RS 800-CX (Kempele, Finland).

Testing of peak oxygen consumption (VO_{2peak}): After the incremental test, participants were allowed 5-10 min of rest before the VO_{2peak} test. The VO_{2peak} test started at the last workload in with blood lactate was below 4 mM during the incremental test. The load was increased by 25 Watts every 60 s until exhaustion. VO_{2peak} was estimated as the highest 1 min average for VO_2. Linear regression was used to establish the relationship between aerobic workload and VO_2 during the incremental test, and the workload corresponding to 70 % of VO_{2peak} was calculated for subsequent testing.

Diet and training before baseline biopsy and interventions

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

*Baseline muscle and blood sampling***:** Participants reported to the laboratory at 8.00 AM after an overnight fast. The muscle biopsy was taken from the vastus lateralis. After removing hair with a razor from the thigh area, the skin was disinfected with Klorhexidine (0.5% SAiD). Then 2 ml Lidocaine (10 $mg \cdot ml^{-1}$) was injected subcutaneously above and beneath the muscle fascia. A small incision (5 mm) was made in the skin and muscle fascia with a scalpel. Any bleeding was stopped by pressure on the wound for approximately 5 min. Biopsies were taken with a Bergström needle modified for suction. The tissue was quickly examined and frozen in liquid nitrogen (-196 °C), and stored at -80 °C until further analysis. A venous blood sample (8 ml heparinized tube) was taken from the v. basilica in a supine position. Blood samples were kept on ice until centrifugation (10 min at 4 °C and 1300 g). After centrifugation, the plasma was pipetted in Eppendorf tubes and stored at -80 °C until further analysis. Lastly, a capillary blood sample was taken for glucose analysis (Hemocue Glucose 201+, Angelholm, Sweden).

Familiarization trial

A preliminary trial was performed to familiarize the participants with cycling and adjusting workload on the SRM ergometer. The trial started with a standardised warm-up consisting of three sets of 4 min cycling at workloads corresponding to 50, 55 and 60% of VO_{2peak}. The same warm up was used in all subsequent testing throughout the study. After a warm up, the workload was set to the estimated workload corresponding to 70% of VO_{2peak}. VO_2 was measured after 4 min over 90 s. If the VO_2 was more than 1 $ml \cdot kg^{-1} \cdot min^{-1}$ from the calculated 70% of VO_{2peak}, workload was adjusted accordingly, and VO_2 was measured 4 min later. The participants were allowed a 15 min break after the workload was adjusted. Afterwards they cycled 30 min at the workload corresponding to 70% of VO_{2peak} ($W_{70\%}$) during which time VO_2, capillary blood samples and rate of perceived exertion (RPE – Borg scale) were

recorded every 10 min. The familiarization trial was completed at least three days after the resting biopsy.

The dietary intervention days

On the two experimental test days, participants reported to the laboratory at 7:30 AM. Initially, resting VO_2, RER and HR were measured for 10 min while the participants were in a supine position (results obtained from the last 5 min). After the 10 min rest, venous and capillary blood samples were taken (Figure 1). The participants were then asked to empty their bladder before the exercise started.

Initial glycogen depleting exercise: On test days the initial exercise session began at 8:00 AM, with the aim of depleting muscle glycogen. The exercise started with a standardized warm up, and after 5 min of rest the participants started cycling at 70% of VO_{2peak}. The exercise was divided into cycling sessions separated by 5 min breaks. The first session lasted 30 min, and all subsequent sessions lasted 20 min. The participants were reminded to drink water every 10 min of cycling. VO_2 and RER were measured over 90 s after 3.5 min of the first session, at the end of each session and the last 60 s before exhaustion. Following measurement of VO_2, and before measurement of capillary lactate and glucose, rating of perceived exertion was record. HR was measured throughout the performance test. Participants cycled until exhaustion at a workload corresponding to 70% of VO_{2peak}. Then after a 5 min rest, a series of 1 min sprints at a workload corresponding to 90% of VO_{2peak}, interspersed with 1 min breaks, were performed until the participants could not maintain their predefined peddling cadence. Capillary glucose and lactate were measured at exhaustion as described above.

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

Muscle biopsies: After the first blood sample, *m. vastus lateralis* was prepared for muscle biopsy as described for the resting biopsy. The 5 h recovery period started when the biopsy

was taken approximately 15 min after the exhaustive exercise protocol. A second biopsy was taken from the opposite leg after the 5-h recovery.

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

Additional measurements during the recovery period: HR was measured during exercise and the 5-h recovery period (Polar Pro trainer 5, Kempele, Finland). Data are presented as means of the first 2 h and the last 3 h of recovery. Resting metabolism was measured after 4.5 h of recovery. VO_2, RER and HR were measured in a supine position over 10 min, and data for the last 5 min used.

Intervention drinks during recovery: The recovery supplementation during the first 2 h following exhaustive exercise and biopsy procedures was either carbohydrate (CHO) or an isocaloric protein with carbohydrate (CHO+PROT) drink. These supplements were provided in a randomized order. Supplementation was given after the first biopsy, and again after 30, 60 and 90 min of recovery. Tissue and blood sampling were always completed before the participants drank any beverage. CHO: The concentration of carbohydrates in CHO was 170 $g \cdot L^{-1}$ (17%). The carbohydrate was a mixture of 85 $g \cdot L^{-1}$ (50%) glucose and 85 $g \cdot L^{-1}$ (50%) maltodextrin. Glucose was from Merck (Darmstadt, Germany) and the maltodextrin from WWR (Herlev, Denmark). Participants were given 0.6 g $CHO \cdot kg^{-1}$ every 30 min during the first 90 min of recovery. Thus, 1.2 g $CHO \cdot kg^{-1} \cdot hr^{-1}$ was ingested during the first 90 min of recovery.
CHO+PROT: The CHO+PROT drink was isocaloric with the CHO drink. The concentration of carbohydrate and protein was 113.7 and 56.3 $g \cdot L^{-1}$, respectively (170 $g \cdot L^{-1}$). Drinks consisted of 56.3 $g \cdot L^{-1}$ glucose, 56.3 $g \cdot L^{-1}$ maltodextrin and 56.3 $g \cdot L^{-1}$ whey protein. The protein was whey isolate protein (Lacprodan, SP-9225 Instant), provided by Arla Food Ingredients P/S (Aarhus, Denmark). Participants were given 0.4 $g \cdot kg^{-1}$ of carbohydrate and 0.2 $g \cdot kg^{-1}$ of whey protein every 30 min during the first 90 min of recovery. Thus, 0.8 g carbohydrate $\cdot kg^{-1} \cdot hr^{-1}$ and 0.4 g protein $\cdot kg^{-1} \cdot hr^{-1}$ were ingested during the first 90 min of recovery. All drinks were served in opaque bottles. To make the drinks comparable in taste, a non-caloric fruit flavoured sweetener and 0.7 mg/L sodium chloride were added to the drinks.

Additional food and recovery supplementation: After 2 h of recovery a meal containing minced meat, pasta and tomato sauce was served according to body weight. The amounts of carbohydrate, protein and fat served in the lunch were 1.7, 0.5 and 0.2 $g \cdot kg^{-1}$, respectively. After 4 h of recovery, a carbohydrate drink (1.2 g carbohydrate$\cdot kg^{-1}$) was provided after both CHO and CHO+PROT treatments.

Performance test

The endurance performance test consisted of cycling until exhaustion at $W_{70\%}$ of VO_{2peak}. After a standardized warm up and 5 min of rest, participants started the performance test. Participants were blinded to time for the duration. VO_2 and RER were measured over 90 s after 3.5 min every 15th min and during the least 60 s before exhaustion. After each VO_2 and RER measurement, participants were asked for their rating of perceived exertion (RPE) followed by drawing blood samples for determination of lactate and glucose. During exercise participants were asked to drink water approximately every 10 min. After the performance test, a third biopsy was taken from the same leg as the first biopsy.

Insulin

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

Western blot

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

Primary antibodies: The following antibodies were from Cell Signalling Technology (Beverly, Ma, USA): GS Ser^{641} – also called GS site 3a (#3891), AMPK Thr^{172} (#2531), PKB Ser^{473} (#9271), GSK-3 Ser^{21} (#9331), p70s6k Thr^{389} (#9205), AS160 Ser^{588} (#8930), TSC2 Thr^{1462} - Tuberin (#3611), and GAPDH - Clone 14C10 (#21185). Antibodies against AMPK-

α2 (sc-19131) and p70s6k Thr/Ser$^{421/424}$ (sc-7984-R) were from Santa Cruz Biotechnology (Dallas, TX, USA). Total GS and site 2+2a have been described and validated by Højlund et al. (19).

Glycogen synthase activity

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

Muscle glycogen

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

Nitrogen balance

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

Exclusion

Ten participants were included in the study. When the last five participants had completed the first dietary intervention, the power-control unit on the SRM ergometer failed to work. With the replacement power-control unit, heart rate was found to be ~20 beats lower and VO_2 ~0.5 $L \cdot min^{-1}$ less of predetermined load compared for the first of the last 5 participants tested. The data for this participant was excluded from all analyses. For the last 4 participants, during their second dietary intervention exercise test, the load on the new SRM ergometer was adjusted to obtain comparable heart rate and VO_2 as occurred during their initial test with the

original power-control. Therefore, the data on the performance test was excluded for these subjects, whereas data from blood and muscle samples were included.

Statistical analysis: Data are presented as mean and SEM. Repeated measurements ANOVA was used to compared measurements during exercise and recovery periods, with Least Significant Difference (LSD) used for post hoc testing. Student's paired t-test was used to compare time to exhaustion and nitrogen balance after CHO+PROT and CHO. $P<0.05$ was considered significant.

RESULTS

Fasted blood glucose was 4.8±0.1, 5.1±0.2 and 4.7± 0.1 mM before the baseline biopsy, CHO and CHO+PROT interventions, respectively. Fasted blood lactate was 0.71±0.08 and 0.88±0.14 mM before CHO and CHO+PROT, respectively. Resting VO_2, measured in a supine position, was 228±15 and 241±15 ml $O_2 \cdot min^{-1}$ before CHO and CHO+PROT, respectively. RER values were 0.96±0.03 and 0.96±0.03 and HR was 49±3 and 51±3 beats$\cdot min^{-1}$ before CHO and CHO+PROT, respectively. No differences in these resting measures were observed between trials ($p>0.05$).

Exercise prior to the dietary interventions: Time to exhaustion at $W_{70\%}$ was similar prior to the CHO and CHO+PROT interventions (107.0±5.6 and 101.7±9.0 min, respectively). During the exercise, VO_2, heart rate, and RPE increased gradually (Table 1). Blood glucose concentration decreased gradually, whereas lactate increased rapidly and remained stable during exercise (Table 1). At exhaustion and after the 1-min sprints, blood glucose was ~ 3.5 mM (Table 1).

Recovery period: Following exercise, blood glucose concentration increased rapidly after intake of CHO and CHO+PROT (Figure 2A). Blood glucose was higher in CHO compared to CHO+PROT during the 90 min dietary intervention (treatment effect) and peaked at 9.1±0.4 mM after 60 min. During the last 3 h of the recovery period, when diet was similar, blood glucose decreased (time effect) and there were no differences between conditions. Plasma insulin increased rapidly following intake of both CHO and CHO+PROT with no significant difference between interventions (Figure 2B). Following exercise, blood lactate fell rapidly

(Figure 1C). HR was similar between CHO and CHO+PROT during the recovery period. After 4.5 h of recovery, resting VO_2 (CHO: 307±19 ml·min^{-1} and CHO+PROT: 311±18 ml·min^{-1}), RER (CHO: 0.91±0.03 in CHO+PROT 0.90±0.02) and heart rate (CHO: 65±3 and CHO+PROT: 65±2 beats·min^{-1}) were similar between CHO and CHO+PROT. Compared to the resting morning values, VO_2 and HR were significantly elevated during the recovery period ($p<0.01$).

Muscle samples: Muscle glycogen concentration was 494.6±29.1 mmol·kg dw^{-1} in the baseline biopsy after an overnight fast (Figure 3A). The exhaustive exercise bouts reduced the muscle glycogen stores to a similar degree with glycogen content being 141.9±29.9 and 106.7±30.7 mmol·kg dw^{-1} prior to CHO and CHO+PROT supplementation, respectively (Figure 3A). During recovery, rate of glycogen synthesis was 35.2±3.8 and 40.9±5.4 mmol·kg dw^{-1}·h^{-1} in CHO and CHO+PROT, respectively (Figure 3C; $p=0.15$ Student's t.-test). Thus, before the performance test, glycogen content was 318.0±32.1 mmol·kg dw^{-1} for CHO and 311.3±32.8 mmol·kg dw^{-1} for CHO+PROT. Glycogen utilization during the exercise before dietary supplementation or during the subsequent performance tests did not differ between the two treatments (Figure 3B). Rates of glycogen synthesis during the two treatments did not differ significantly (Figure 3C). Rates of glycogen synthesis during the two treatments were significantly correlated (Figure 3D; $r=0.75$; $p<0.02$). Phosphorylation of AMPK at Thr^{172} increased after both exercise sessions, with no effect of the dietary treatments found (Figure 3E). Glycogen synthase activity was investigated and all parameters (GS $FV_{0.1}$, GS $FV_{1.67}$ and GS %-I-form) were activated after exercise to a similar degree prior to the dietary treatments (Figure 4A-C). Interestingly, GS remained activated during the 5 h recovery despite a high rate of glycogen synthesis (Figure 4A-C). Phosphorylation of GS at site 3 and site 2+2a was reduced after exercise and remained low during the 5 h recovery, and agreed with the GS activity data. GS total activity was not influenced by the exercise or dietary treatments as expected (Figure 4E-F). GSK-3 phosphorylation was not influenced by exercise or dietary intervention (Figure 4G).

Phosphorylation of Akt at Ser^{473} increased immediately after exercise and phosphorylation was elevated further after 5 h of recovery, but no differences were found between CHO and CHO+PROT (Figure 5A). Phosphorylation of p70S6K at Thr^{389} was not increased immediately after exercise, but phosphorylation was elevated similarly in CHO and CHO+PROT after 5 h (Figure 5B). Phosphorylation of p70S6K at Thr^{421}/Ser^{424} did not change during the dietary intervention (Figure 5C). Likewise, TSC2 Thr^{1462} phosphorylation

was unchanged at the time points studied (Figure 5D). Phosphorylation of TBC1D4/AS160 Ser^{588} increased immediately after exercise and remained elevated to a similar level in CHO and CHO+PROT (Figure 5E).

Performance test: Time to exhaustion at $W_{70\%}$ lasted on average 8.4±1.8 min longer in CHO+PROT than CHO (Figure 6; $p<0.009$; n=5). Following the performance test protocol, muscle glycogen was reduced equally to 152.2±32.0 and 157.1±34.0 $mmol \cdot kg \cdot dw^{-1}$ in CHO and CHO+PROT, respectively (Figure 6).

Data of VO_2, RER, HR and RPE from the performance test are summarized in Figure 7. VO_2, RER, heart rate, and RPE increased during the performance test, but there were no differences between the two dietary interventions. Blood glucose declined during the first min of exercise, but returned to basal level at exhaustion (Figure 7C). Lactate increased to ~2 mM during exercise and there were no differences between CHO and CHO+PROT (Figure 7D).

Nitrogen balance: Net nitrogen balance was positive in CHO+PROT (19.6±7.6 $mg \cdot kg^{-1}$; p=0.04). Nitrogen balance was not significantly different from zero in CHO (-10.7±6.3 $mg \cdot kg^{-1}$; p=0.22) but significantly lower than CHO+PROT (p=0.009). Nitrogen excretion was higher in CHO+PROT than in CHO (9.9±0.5 vs 7.4±0.6 g; p=0.008) during the recovery period. Urea nitrogen accounted for 92.2 ±1.3 and 91.9±0.8% of total urine nitrogen excretion in CHO and CHO+PROT, respectively.

DISCUSSION

The present study is one of the first where muscle biopsies have been taken in a setting where endurance performance is improved after co-ingestion of protein and carbohydrate compared with intake of carbohydrate only during the first two hours of the recovery period. The rate of glycogen synthesis and activation of anabolic signalling molecules during the recovery period were not noticeably different between CHO and CHO+PROT. Although performance improved after co-ingestion of protein and carbohydrate compared with carbohydrate only, glycogen degradation and activation of signalling molecules during the performance tests were not significantly different between treatments. On the other hand, nitrogen balance was

positive only after co-ingestion of protein and carbohydrate and may have contributed to optimising recovery of performance after exhaustive exercise.

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

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

As mentioned previously, we did not find a significant difference in rate of glycogen synthesis between the interventions, despite reports that co-ingestion of protein and carbohydrates increased the rate of glycogen synthesis more than after intake of carbohydrate only (5; 70). Indeed, not all studies report elevated rates of glycogen synthesis after exercise when protein and carbohydrates are co-ingested (28; 62). However, the rate of glycogen synthesis is difficult to study because of variation in glycogen content in muscle biopsies. In a study by Jentjens et al., glycogen content after the exercise before CHO and CHO+PROT interventions were 106±19 and 176±31 mmol·kg dw^{-1}, respectively, but rate of glycogen synthesis was numerically lower in CHO than CHO+PROT (225±22 vs 252 ±48 mmol·kg dw^{-1}). This is

surprising, because low glycogen content activates glycogen synthase and stimulates glycogen synthesis (24; 33-35).

In the present study glycogen content was 142±30 mmol·kg dw^{-1} and 107±31 prior to CHO and CHO+PROT, respectively, and rate of glycogen synthesis was numerically higher after CHO+PROT compared to CHO ($p=0.15$; two-tailed t-test). However, this tendency for a higher rate of glycogen synthesis in CHO+PROT could have been influenced by the numerically lower post-exercise glycogen content in CHO+PROT compared to CHO (25). Moreover, rates of glycogen synthesis during the two dietary interventions (CHO+PROT and CHO) were significantly correlated ($r=0.75$; $p<0.02$; Figure Fig 3D), which suggests that inter-individual variation (genetic or training status) determined the rate of glycogen synthesis rather than the treatment provided.

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

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

Hypoglycemia can also result in fatigue during prolonged endurance exercise (10). However, we observed no signs of hypoglycemia during the performance test after the dietary

interventions, and the decline in blood glucose was more pronounced during the exhaustive exercise prior to the dietary interventions. This supports our previous findings that mechanisms other than blood glucose availability limits performance after recovery from exhaustive exercise (54). Importantly, the glycogen content at exhaustion was similar in the exercise session before and after the dietary interventions and independent of treatment indicating that low glycogen content contributed to fatigue in all conditions. The fact that the increase in AMPK phosphorylation did not differ between treatments or the two exercise sessions is not surprising because the participants cycled until exhaustion and similar metabolic stress may have developed and caused fatigue (31; 67).

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

In the present study, nitrogen balance was positive in CHO+PROT with intake of 1.3 g protein/kg during the recovery period. By contrast, nitrogen balance was negative for CHO, during which only 0.5 g protein/kg was ingested during the 5 h recovery. Endurance athletes

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

A most important finding in the present study was that glycogen synthase fractional activity remained highly activated without any decline during the 5 h recovery. High glycogen content normally inhibits GS activity (11; 24), and the finding that ~200 mmol·kg^{-1} glycogen can be synthesised without any reduction in GS activity is remarkable. In this regard, muscle contraction increases GS activity via dephosphorylation of GS 3 sites and site 2+2a (33; 34). In the present study, phosphorylation of GS at site 3a and site 2+2a remained low during the 5 h recovery explaining the high activity. GSK3, which phosphorylates GS at the 3-sites, was not regulated by exercise as expected (68). Exercise-induced activation of GS requires the protein phosphatase-1 binding subunit R_{GL} (*PPP1R3A*), suggesting that activation of protein phosphatase is required for exercise to activate GS (2). Recently, we showed that glycogen synthase activity was higher than expected for the glycogen content established 24 h after exercise (18). We can now expand this finding by showing that GS remains activated despite substantial glycogen synthesis during the first 5 h of recovery. Our recent study showed that AMPK is required for glycogen supercompensation (18). Although AMPK phosphorylation was not elevated after 5 h of recovery, our recent study showed that the $\alpha_1\beta_2\gamma_1$ subunit activity remained elevated even after AMPK phosphorylation had returned to basal level (18). Viewed comprehensively, these results suggest that GS activity can become decoupled from glycogen-mediated inhibition, and may help explain how glycogen content can supercompensate after exhaustive exercise (7; 18).

It is a limitation of the present study that no muscle biopsies were taken during the first part of the dietary intervention when either protein and carbohydrate or carbohydrate only were supplied. This may explain why no significant differences in activation of anabolic signalling

were observed between treatments. The small sample size, in particular for performance, but our power analysis indicated the participant number was adequate. Finally, the study only included well-trained males, which preclude generalisation of the results.

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

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LEGENDS

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

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

Figure 3. Glycogen metabolism and phosphorylation of AMPK during the interventions. (A) Glycogen content in muscles before exercise, after the exercise prior to the dietary interventions, after 5 h recovery and after the performance test. (B) Glycogen breakdown during the exercise prior to the dietary intervention and during the performance test. (C) Glycogen synthesis during the recovery period. (D) Correlation between glycogen synthesis during the 5 h recovery period with the two dietary interventions (CHO and CHO+PROT). (E) AMPK Thr^{172} phosphorylation in muscles before exercise, after the exercise prior to the dietary interventions, after 5 h recovery and after the performance test. (F) Representative blots. Data are mean±SEM. N=8-9. See Methods for description of the antibodies.

Abbreviation: B: Basal before exercise; EX1: Exercise before dietary intervention; RE: After 5 h recovery; EX2: Performance test after the dietary intervention; CHO: Carbohydrate intake during the first 2 h recovery; CHO+PROT: Carbohydrate plus protein during the first 2 h recovery; a: $p<0.05$ compared to Pre; b: $p<0.05$ compared to 5 h Recovery; *: $p<0.05$ compared to EX1.

Figure 4. Glycogen synthase activity and phosphorylation during the intervention. (A) Glycogen synthase fractional activity in the presence of 0.1 mM UDP-glucose (GS $FV_{0.1}$) in muscles before exercise, after the exercise prior to the dietary interventions, after 5 h recovery and after the performance test. (B) Glycogen synthase fractional activity in the presence of 1.67 mM UDP-glucose (GS $FV_{1.67}$) in muscles before exercise, after the exercise prior to the dietary interventions, after 5 h recovery and after the performance test. (C) Glycogen synthase I-form in muscles before exercise, after the exercise prior to the dietary interventions, after 5 h recovery and after the performance test. (D) Total glycogen synthase activity in muscles before exercise, after the exercise prior to the dietary interventions, after 5 h recovery and after the performance test. (E) Glycogen synthase phosphorylation at site 2+2a in muscles before exercise, after the exercise prior to the dietary interventions, after 5 h recovery and after the performance test. (F) Glycogen synthase phosphorylation at site 3 in muscles before exercise, after the exercise prior to the dietary interventions, after 5 h recovery and after the

performance test. (G) GSK3β Ser^{21} phosphorylation in muscles before exercise, after the exercise prior to the dietary interventions, after 5 h recovery and after the performance test. See Fig 3 for blots. Data are mean±SEM. N=8-9

Abbreviations: GS: Glycogen synthase; GS $FV_{0.1}$: Fractional activity with 0.1 mM UDP-glucose in assay buffer; Glycogen synthase; GS $FV_{1.67}$: Fractional activity with 1.67 mM UDP-glucose in assay buffer; GS I-form: Fractional activity with 1.67 mM UDP-glucose and 0.01 mM glucose 6-phosphate; pGS: glycogen synthase phosphorylation; a: $p<0.05$ compared to Pre; b: $p<0.05$ compared to 5 h Recovery.

Figure 5. Phosphorylation of signalling molecules during the interventions. Muscle biopsies were taken before exercise, after the exercise prior to the dietary interventions, after 5 h recovery and after the performance test for measurement of Akt Ser^{473} (A), p70S6K Thr^{389} (B), p70S6K Thr/$Ser^{421/424}$ (C), TCS2 Thr^{1462} (D) and AS160 Ser^{588} (E) phosphorylation. See Fig 3 for blots. Data are mean±SEM. N=8-9

Abbreviation: See Material and methods for full names of proteins. a: $p<0.05$ compared to Pre; b: $p<0.05$ compared to 5 h recovery; d: $p<0.05$ compared Post EX2.

Figure 6. Performance time to exhaustion after intake of CHO+PROT or CHO during the first 2 h of the 5 h recovery period. Data are mean±SEM; N=5. *: $p<0.05$ compared to CHO.

Figure 7. Oxygen uptake, respiratory exchange ratio, glucose, lactate, heart rate and perceived exertion during the endurance performance test after 5 h of recovery. Data are mean±SEM. N=9.

Figure 1

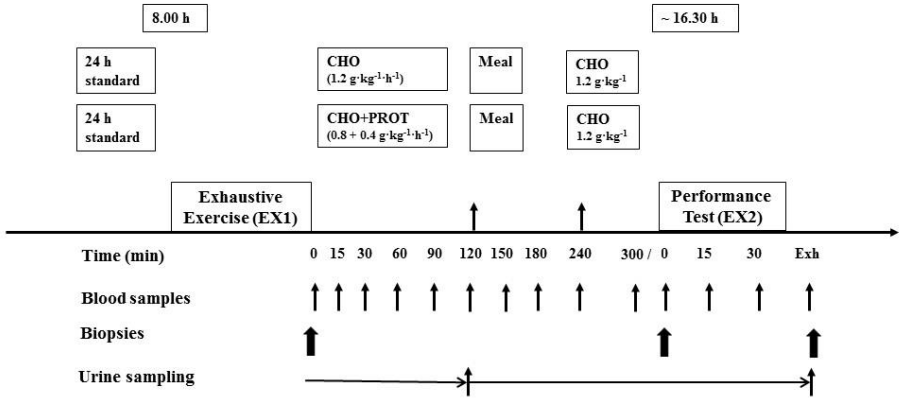

8.00 h
~ 16.30 h
24 h standard
24 h standard
CHO (1.2 g·kg-1·h-1)
CHO+PROT (0.8 + 0.4 g·kg-1·h-1)
Meal
Meal
CHO 1.2 g·kg-1
CHO 1.2 g·kg-1
Exhaustive Exercise (EX1)
Performance Test (EX2)
Time (min)
0 15 30 60 90 120 150 180 240 300 / 0 15 30 Exh
Blood samples
Biopsies
Urine sampling

Figure 2

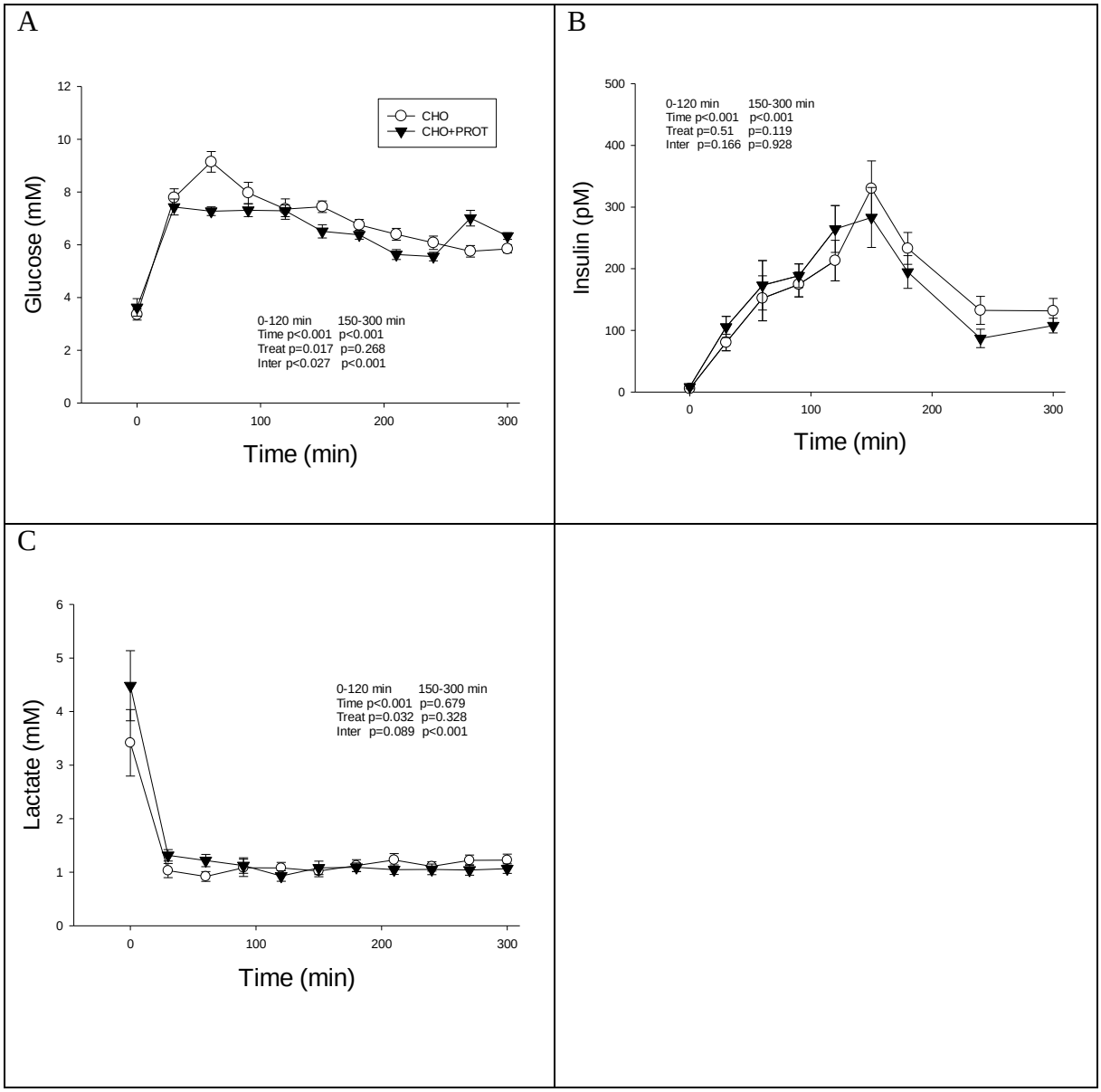

A
Glucose (mM)
Time (min)
CHO
CHO+PROT
0-120 min 150-300 min
Time p<0.001 p<0.001
Treat p=0.017 p=0.268
Inter p<0.027 p<0.001
B
Insulin (pM)
Time (min)
0-120 min 150-300 min
Time p<0.001 p<0.001
Treat p=0.51 p=0.119
Inter p=0.166 p=0.928
C
Lactate (mM)
Time (min)
0-120 min 150-300 min
Time p<0.001 p=0.679
Treat p=0.032 p=0.328
Inter p=0.089 p<0.001

Figure 3

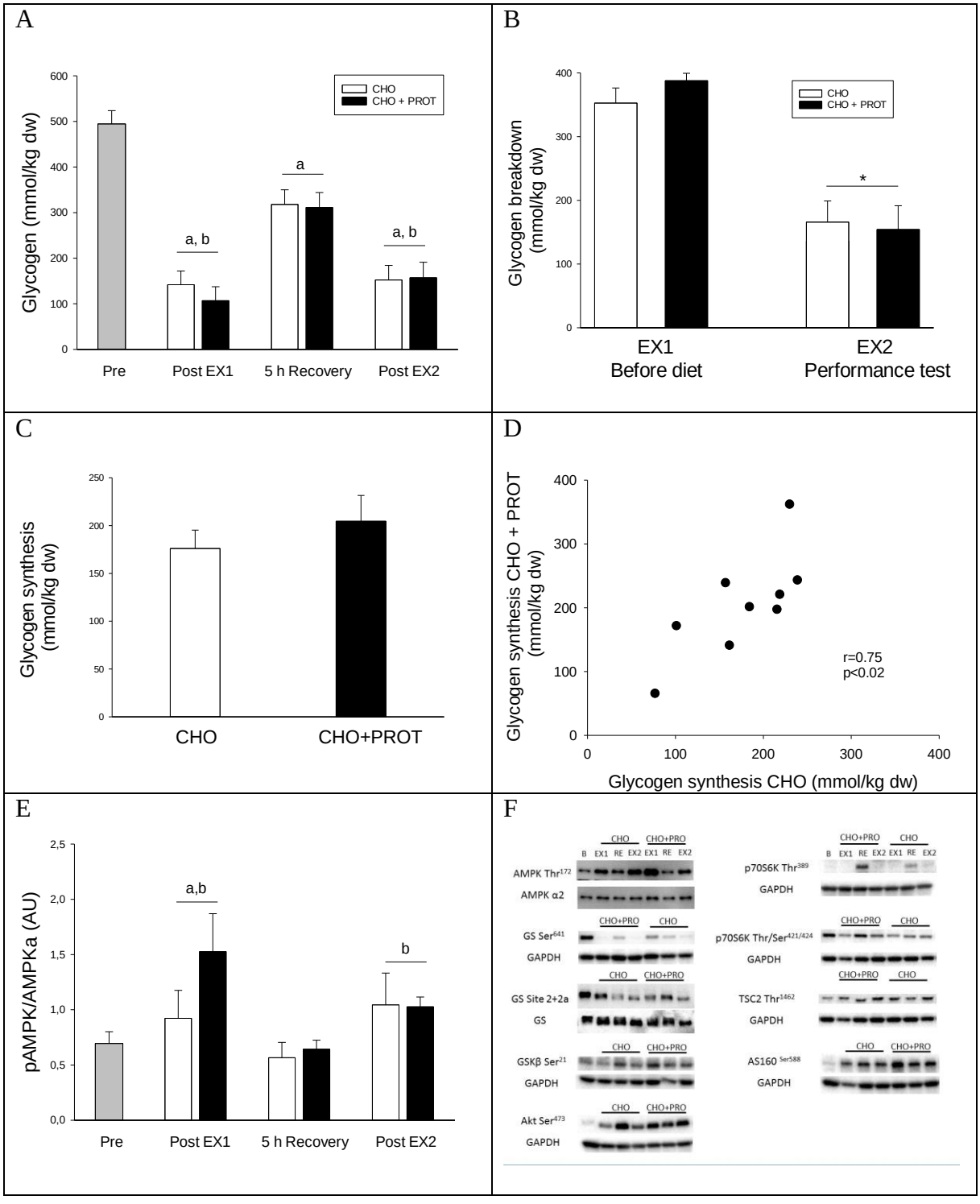

A
Glycogen (mmol/kg dw)
CHO
CHO + PROT
a, b
a
a, b
Pre
Post EX1
5 h Recovery
Post EX2
B
Glycogen breakdown (mmol/kg dw)
CHO
CHO + PROT
*
EX1
Before diet
EX2
Performance test
C
Glycogen synthesis (mmol/kg dw)
CHO
CHO+PROT
D
Glycogen synthesis CHO + PROT (mmol/kg dw)
r=0.75
p<0.02
Glycogen synthesis CHO (mmol/kg dw)
E
pAMPK/AMPKa (AU)
a,b
b
Pre
Post EX1
5 h Recovery
Post EX2
F
AMPK Thr172
AMPK α2
GS Ser641
GAPDH
GS Site 2+2a
GS
GSKβ Ser21
Akt Ser473
p70S6K Thr389
p70S6K Thr/Ser421/424
TSC2 Thr1462
AS160 Ser588

Figure 4

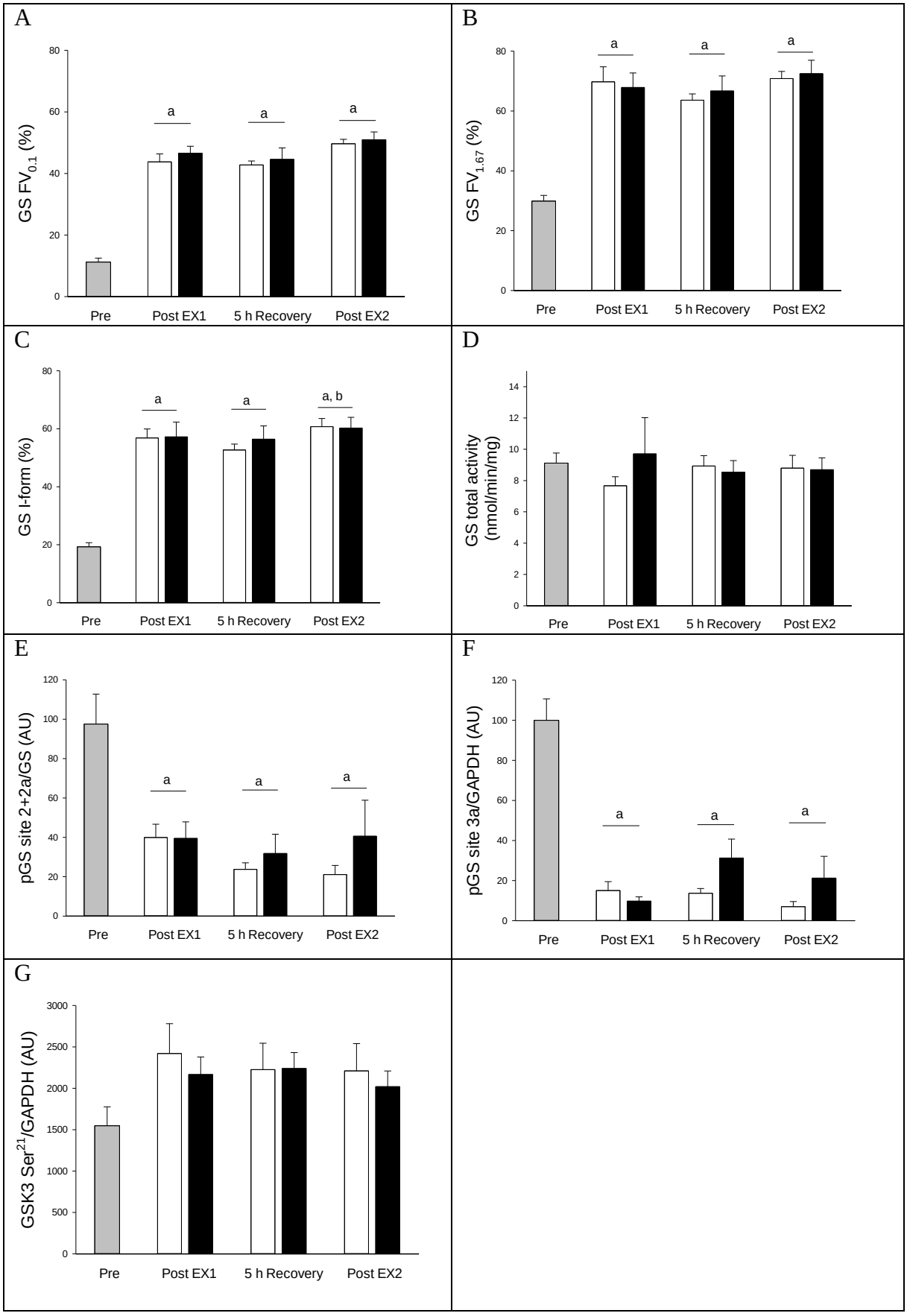

A
GS FV0.1 (%)
a
a
a
Pre
Post EX1
5 h Recovery
Post EX2
B
GS FV1.67 (%)
a
a
a
Pre
Post EX1
5 h Recovery
Post EX2
C
GS I-form (%)
a
a
a, b
Pre
Post EX1
5 h Recovery
Post EX2
D
GS total activity (nmol/min/mg)
Pre
Post EX1
5 h Recovery
Post EX2
E
pGS site 2+2a/GS (AU)
a
a
a
Pre
Post EX1
5 h Recovery
Post EX2
F
pGS site 3a/GAPDH (AU)
a
a
a
Pre
Post EX1
5 h Recovery
Post EX2
G
GSK3 Ser21/GAPDH (AU)
Pre
Post EX1
5 h Recovery
Post EX2

Figure 5

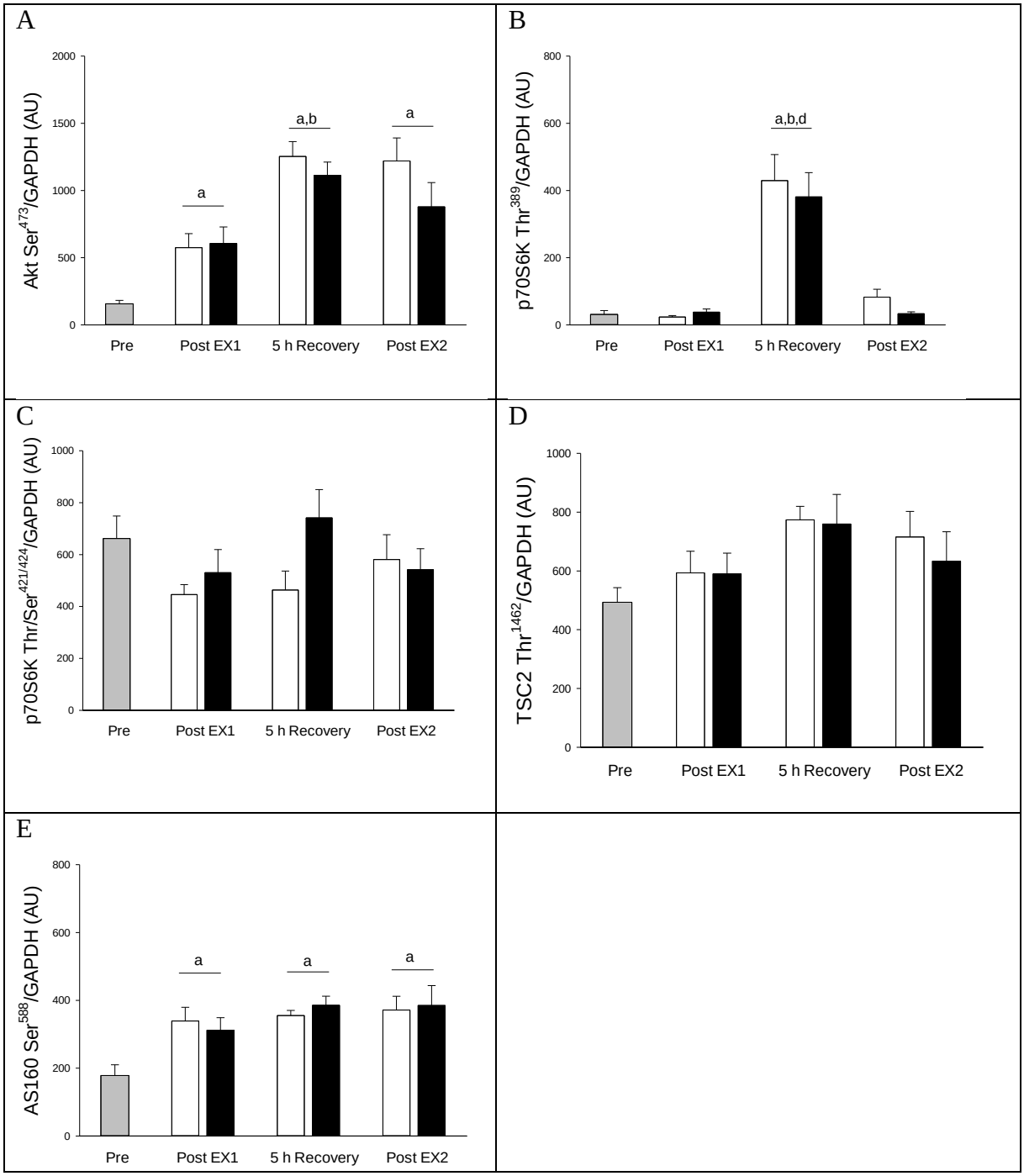

A
Akt Ser473/GAPDH (AU)
2000
1500
1000
500
0
a
a,b
a
Pre
Post EX1
5 h Recovery
Post EX2
B
p70S6K Thr389/GAPDH (AU)
800
600
400
200
0
a,b,d
Pre
Post EX1
5 h Recovery
Post EX2
C
p70S6K Thr/Ser421/424/GAPDH (AU)
1000
800
600
400
200
0
Pre
Post EX1
5 h Recovery
Post EX2
D
TSC2 Thr1462/GAPDH (AU)
1000
800
600
400
200
0
Pre
Post EX1
5 h Recovery
Post EX2
E
AS160 Ser588/GAPDH (AU)
800
600
400
200
0
a
a
a
Pre
Post EX1
5 h Recovery
Post EX2

Figure 6

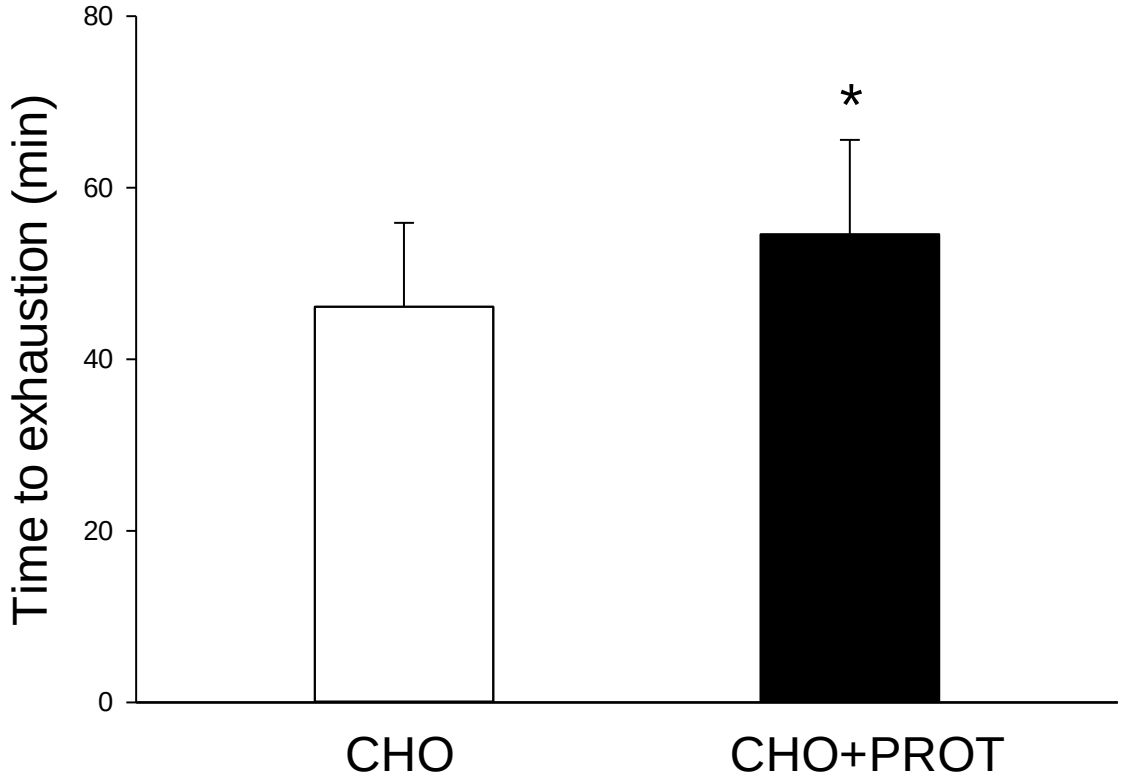
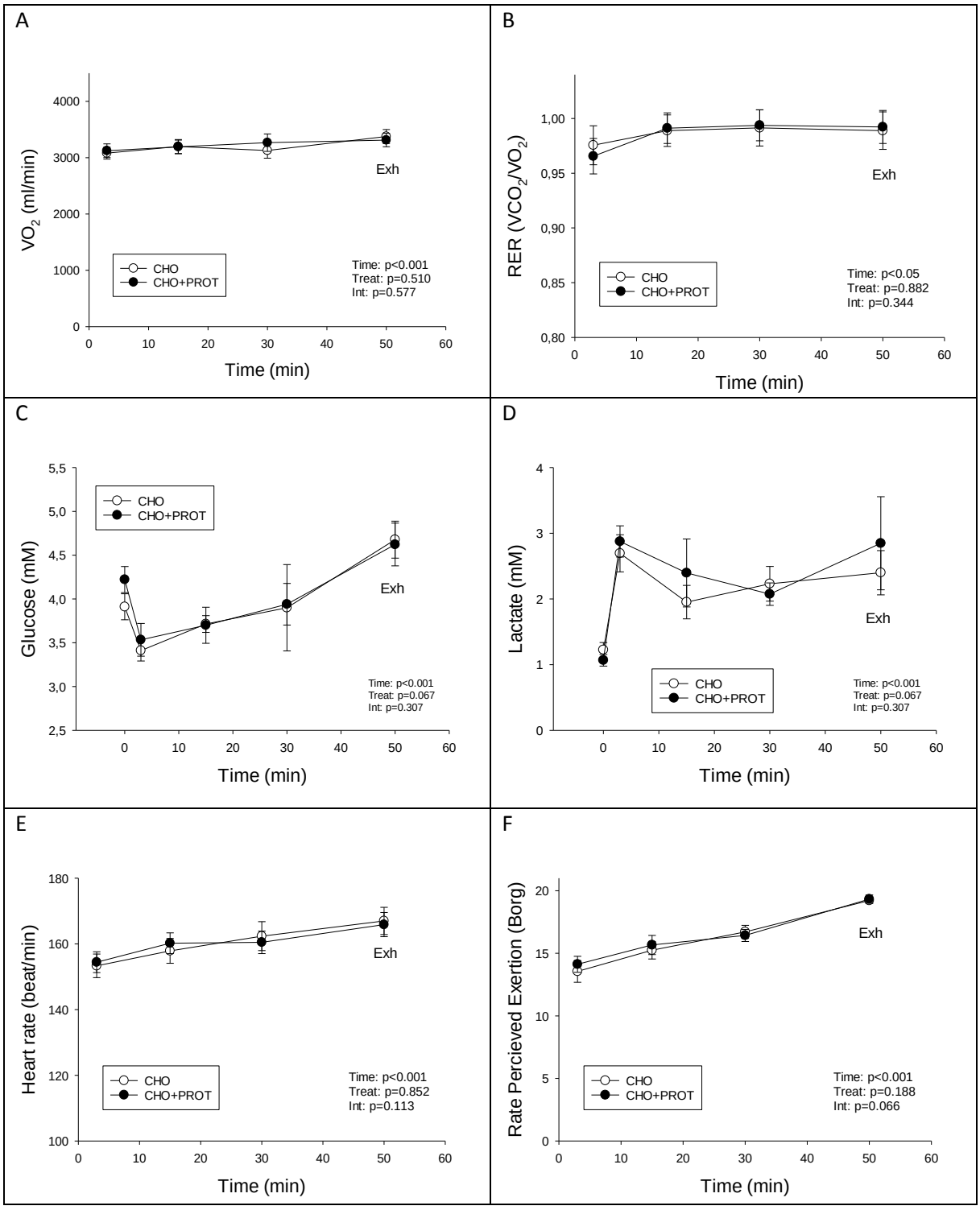


Figure 7


A
VO_2 (ml/min)
4000
3000
2000
1000
0
0 10 20 30 40 50 60
Time (min)
Exh
CHO
CHO+PROT
Time: p<0.001
Treat: p=0.510
Int: p=0.577
B
RER (VCO_2/VO_2)
1,00
0,95
0,90
0,85
0,80
0 10 20 30 40 50 60
Time (min)
Exh
CHO
CHO+PROT
Time: p<0.05
Treat: p=0.882
Int: p=0.344
C
Glucose (mM)
5,5
5,0
4,5
4,0
3,5
3,0
2,5
0 10 20 30 40 50 60
Time (min)
Exh
CHO
CHO+PROT
Time: p<0.001
Treat: p=0.067
Int: p=0.307
D
Lactate (mM)
4
3
2
1
0
0 10 20 30 40 50 60
Time (min)
Exh
CHO
CHO+PROT
Time: p<0.001
Treat: p=0.067
Int: p=0.307
E
Heart rate (beat/min)
180
160
140
120
100
0 10 20 30 40 50 60
Time (min)
Exh
CHO
CHO+PROT
Time: p<0.001
Treat: p=0.852
Int: p=0.113
F
Rate Percieved Exertion (Borg)
20
15
10
5
0
0 10 20 30 40 50 60
Time (min)
Exh
CHO
CHO+PROT
Time: p<0.001
Treat: p=0.188
Int: p=0.066