Amino Acids (2015) 47:767–778
DOI 10.1007/s00726-014-1907-8

ORIGINAL ARTICLE

No differential effects of divergent isocaloric supplements on signaling for muscle protein turnover during recovery from muscle-damaging eccentric exercise

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Received: 3 July 2014 / Accepted: 17 December 2014 / Published online: 6 January 2015

Abstract Unaccustomed high-intensity eccentric exercise (ECC) can provoke muscle damage including several days of muscle force loss. Post-exercise dietary supplementation may provide a strategy to accelerate rate of force regain by affecting mechanisms related to muscle protein turnover. The aim of the current study was to investigate if protein signaling mechanisms involved in muscle protein turnover would be differentially affected by supplementation with either whey protein hydrolysate and carbohydrate (WPH+CHO) versus isocaloric carbohydrate (CHO) after muscle-damaging ECC. Twenty-four young healthy participants received either WPH+CHO ($n = 12$) or CHO supplements ($n = 12$) during post-exercise recovery from 150 maximal unilateral eccentric contractions. Prior to, at 3 h and at 24, 48, 96 and/or 168 h post-exercise, muscle strength, muscle soreness, and Akt-mTOR and FOXO signaling proteins, were measured in an ECC exercising leg and in the contralateral non-exercise control leg (CON). After ECC, muscle force decreased by 23–27 % at 24 h post-exercise, which was followed by gradual, although not full recovery at 168 h post-exercise, with no differences between supplement groups. Phosphorylation of mTOR, p70S6K and rpS6 increased and phosphorylation of FOXO1 and FOXO3 decreased in the ECC leg, with no differences between supplement groups. Phosphorylation changes were also observed for rpS6, FOXO1 and FOXO3a in the CON leg, suggesting occurrence of remote tissue effects. In conclusion, divergent dietary supplementation types did not produce differences in signaling for muscle turnover during recovery from muscle-damaging exercise.

Keywords Whey protein · Eccentric exercise · Recovery · mTOR signaling · MuRF1 · FOXO1/3A signaling

Handling Editor: E. Rawson.

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Introduction

High-intensity eccentric contractions (ECC) are known to inflict transient muscle damage, as judged by, e.g., decreases in muscle force capacity (Howell et al. 1993; Vissing et al. 2008), increased concentrations of plasma muscle enzymes and increased muscle soreness (Clarkson et al. 1992; Cooke et al. 2010; Jackman et al. 2010). Specifically, with reference to muscle force production, approximately 15–60 % decreases in the maximal voluntary contraction (MVC) have commonly been observed during the immediate 1–2 days after ECC, depending on the volume and intensity of specific eccentric protocols utilized (Hyldahl and Hubal 2014; Paulsen et al. 2007). This is then typically followed by a fairly linear recovery of muscle force, often not with full recovery even 7 days after exercise (Clarkson et al. 1992; Hyldahl and Hubal 2014; Vissing et al. 2008).

The negative affect on muscle force generating capacity is believed to partly adhere to localized ultrastructural damage to contractile units of the affected muscle fibers, as evidenced by localized Z-band streaming (Feasson et al. 2002; Hortobagyi et al. 1998). Subsequent restoration of exercise-induced ultrastructure myofibrillar damage will then expectedly require degradation of permanently

damaged proteins as well as regeneration and/or de novo synthesis of sarcomeric protein to precondition against re-exposure (Nedergaard et al. 2007). In accordance, previous investigation support that signaling mechanisms involved in protein turnover (i.e., signaling mechanisms involved in protein degradation and protein synthesis, respectively), are activated during recovery from muscle damaging exercise (Eliasson et al. 2006; Stefanetti et al. 2014).

Since loss of muscle force and muscle function after ECC can be both pronounced and long-lasting, strategies to accelerate muscle force regain and/or aid mechanisms involved in muscle regeneration, are of interest for muscle performance. Furthermore, such investigation may contribute to the knowledge on how to counteract muscle wasting secondary to inactivity, disease and/or aging. In this regard, supplementation with protein during post-exercise recovery from muscle-damaging ECC has recently been suggested to reduce the magnitude and duration of strength decline (Buckley et al. 2010; Cooke et al. 2010) and muscle soreness (Jackman et al. 2010). However, whereas two of these studies observe attenuation in muscle force loss with whey protein supplements rich in branched chain amino acids (BCAA) (Buckley et al. 2010; Cooke et al. 2010), another study utilizing BCAA supplementation, observed no effect on recovery of muscle force (Jackman et al. 2010), so the impact of added dietary supplements remains equivocal at this point (Pasiakos et al. 2014; Wojcik et al. 2001). Other than differences in exercise protocols, differences in results between studies likely derive from different compositions of supplements. In this regard, the BCAA, leucine, may be of special interest, since it has been demonstrated to constitute a direct stimulator of the anabolic signaling through the mammalian target of rapamycin (mTOR)-pathway (Anthony et al. 2000; Churchward-Venne et al. 2012; Moberg et al. 2014).

None of the aforementioned studies on post-exercise supplementation after ECC have included investigation on potential underlying myocellular mechanisms that may potentially drive regenerative processes to potentially influence rate of recovery of muscle strength. However, when turning to studies on the effects of dietary supplementation after resistance exercise by hypertrophy principles (i.e., utilizing a lower total volume × intensity of exercise, and not intending to provoke major muscle damage), it is well accepted that protein supplementation stimulates Akt-mTORC1 signaling systems and protein synthesis (Atherton and Smith 2012). In this regard, one should recall that commercially available dietary supplements often contain both glucose and protein. This is interesting because both glucose and protein possess insulinotrophic potential (Gannon and Nuttall 2010), thereby both possessing ability to activate mTORC1 and downstream signaling through an PI3K-Akt axis (Fujita et al. 2007), whereas mTOR may also be directly activated by BCAA independently of an PI3K-Akt axis (Hamilton and Baar 2008). Thus, the composition of dietary supplements may determine the relative magnitude and the signaling paths taken during anabolic signaling (Gannon and Nuttall 2010). As for atrophy signaling, this is also affected by activation of the PI3K-Akt pathway, with Akt inhibiting the fork head transcription factors (FOXO) from promoting expression of E3 ligases such as atrogin-1 and muscle ring finger-1 (MuRF1) (Sandri et al. 2004; Stitt et al. 2004). In accordance, recent findings of Borgenvik et al. (2012) indicate that BCAA supplementation can attenuate MURF1 expression. Thus, protein and/or glucose supplementation may influence signaling systems involved in protein turnover, thereby affecting regeneration and/or remodeling of muscle contractile protein and regain of muscle strength after muscle-damaging exercise. However, the comparative effects of divergent supplement types on signaling for protein turnover and restoration of muscle force generating capacity after ECC, has not been investigated.

The aim of this study was, therefore, to investigate the effects of leucine-rich whey protein hydrolysate and carbohydrate (WPH+CHO) versus isocaloric carbohydrate (CHO) supplementation on the Akt-mTOR and the Akt-FOXO signaling axis, during recovery from muscle-damaging exercise.

We hypothesized that WPH+CHO would accentuate signaling for protein synthesis and attenuate signaling for protein degradation, compared to isocaloric CHO.

Materials and methods

Participants

Twenty-four young healthy recreationally active men volunteered to participate in the study. All subjects were informed of the purpose and risks of the study and gave written, informed consent to participate. The study was approved by The Central Denmark Region Committees on Health Research Ethics (ref. no. M-20110179) and performed in accordance with the Declaration of Helsinki. Exclusion criteria were; (1) participation in systematic resistance training or eccentric dominated activities for lower extremity muscles within 6 months prior to inclusion in the study; (2) a history of lower extremity musculoskeletal injuries; (3) vegan diet, and; (4) use of dietary supplements or prescription medicine that would potentially affect muscle recovery or function (e.g. protein supplements, antioxidants supplements, NSAIDs, angiotensin-converting enzyme inhibitors). All subjects were instructed to refrain from strenuous physical activity 48 h before the exercise trial day and throughout the testing period. Furthermore,

subjects were asked to refrain from taking any type of non-steroidal anti-inflammatory drugs or alcohol during the entire experimental protocol.

Study design

The study was conducted in a double blinded, isocaloric placebo-controlled fashion in regards to dietary supplementation. Following inclusion, subjects were randomly allocated into either a whey protein hydrolysate+carbohydrate group (WPH+CHO, $n = 12$) or isocaloric carbohydrate placebo group (CHO, $n = 12$). Subject anthropometrics were recorded on a separate initial visit to the laboratory [values are mean and 95 % confidence intervals. WPH+CHO-group: height (cm): 181.8 (177.5; 186.1), bodymass (kg): 74.2 (67.7; 80.7), age (years): 23 (21.1; 23.9), bodyfat (%): 12.3 (9.3; 15.3), pre-MVC (Nm): 295.2 (276; 314). CHO-group: height (cm): 181.8 (178.1; 185.4), bodymass (kg): 76.8 (71.1; 82.5), age (years): 24 (22.3; 25.7), bodyfat (%): 14.5 (11.7; 17.2), pre-MVC (Nm): 289.8 (254; 326)]. No differences were observed between the two supplement groups with regards to anthropometric measures. During the initial visit to the laboratory, the settings for the isokinetic dynamometer for each person were also determined.

A schematic overview of the study protocol is presented in Fig. 1. Fourteen days (day -14) prior to the exercise trial day (day 0), the subjects reported to the laboratory between 8.00 and 10.00 am after an overnight fast (from 10 pm the day before). Subjects rested in supine position for 45 min before a muscle biopsy was obtained from the non-exercise leg (randomly selected as either preferred or non-preferred leg). On the exercise trial day, subjects reported to the laboratory at 07.30 am in a fasted state. Before the eccentric exercise protocol was initiated, muscle soreness was evaluated using a visual analog scale, a blood sample was collected and knee extensor muscle contractile function was evaluated. Subsequently, a unilateral eccentric exercise protocol for was completed, lasting approximately 30 min (see below). Immediately after the exercise bout, the subjects ingested either a WPH+CHO or a CHO supplement, according to the group they were assigned, and then rested for 3 h. At 3-h post-exercise, a biopsy was obtained from both the exercise and the non-exercise control leg. Before leaving the laboratory, the subjects ingested the second drink (1.00 pm) and received a third drink to ingest 3 h later (4.00 pm). On days 1 and 2 (24 and 48 h following exercise, respectively), the subjects were instructed to ingest three supplements at absolute time points similar to day 0, with the first drink always ingested after the functional tests and biopsy sampling. Biopsy sampling from both the ECC and the CON leg on days 1 and 2 were performed under conditions similar to the pre-exercise biopsy, i.e., the subjects fasted overnight and rested in the supine position for 45 min prior to biopsy sampling. The biopsy sampling on days 1 and 2 was timed to correspond to 24 and 48 h following exercise termination. Assessments on indices of muscle damage (muscle force, muscle soreness and plasma muscle creatine kinase) were repeated at 24, 48, 72, 96, and 168 h after overnight fasting. Subjects were instructed to refrain from physical activity such as exercise, stair case walking and other types of strenuous activity in the hours/days between post-exercise assessments of indices of muscle damage.

Exercise protocol

The unilateral exercise protocol consisted of 15 × 10 repetitions of maximal isokinetic eccentric contractions for

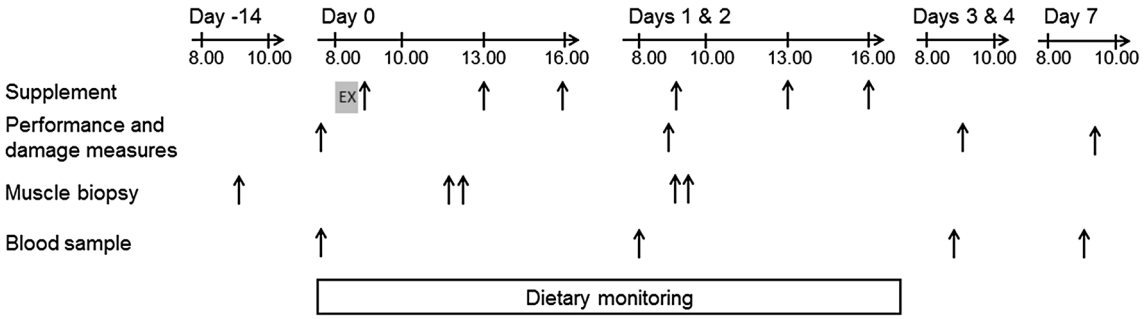

Fig. 1 Timeline of interventions and measurements on the four study days are shown. A muscle biopsy was sampled 14 days prior to the exercise trial (i.e. to establish basal level). The protocol for days 1 and 2 was identical. Performance and damage measures included maximal muscle strength, blood samples for serum creatine kinase analysis and muscle soreness. A biopsy was collected from one randomly selected leg prior to exercise and from both the exercise- and the non-exercise leg at 3, 24 and 48 h during the post-exercise recovery. Exercise (EX) was conducted as 15 × 10 maximal isolated isokinetic eccentric knee extensions. Supplements were ingested three times per day (~9.00 am, 1 pm, and 4 pm) on days 0, 1 and 2 and contained hydrolysed whey protein+carbohydrate (WPH+CHO) or isocaloric carbohydrate placebo (CHO). Dietary monitoring was performed of all energy containing food and drinks consumed during day 0, 1 and 2 after exercise

the knee extensors performed in an isokinetic dynamometer (Humac Norm, CSMI, Stoughton, USA), which is comparable to previous studies (Crameri et al. 2004, 2007). Knee joint range of motion was set at 70 dg and contraction velocity at 30 dg/s to ensure standardized conditions for all subjects and thereby comparability between groups in total work performed during exercise. Individual dynamometer settings were identical to settings during muscle contractile function testing. During exercise, subjects received standard verbal and visual feedback and encouragement to ensure maximal effort. Exercise repetitions and sets were interspaced by 3 and 60 s of recovery, respectively. Force and work data recorded during exercise were saved for later analysis.

Muscle biopsies

Muscle biopsies were obtained under local anesthesia (10 mg/ml lidocaine) from the middle section of the M. vastus lateralis by Bergstrom needle technique as described previously (Vissing et al. 2013). The muscle tissue was immediately quickly dissected free of visible fat and connective tissue and snap frozen in liquid nitrogen and stored at −80 °C until further analysis. It was attempted to collect all biopsies at similar depth of the muscle and to disperse incision holes at least 3 centimeters apart to minimize any potential effect of previous biopsies (Vissing et al. 2005). By similar procedure, biopsies corresponding to all post-exercise time points were also obtained from the non-exercise control leg, to control for systemic effects (Vissing et al. 2005).

Diet and supplementation

Subjects recorded all energy containing food and drinks on days 0, 1 and 2 after exercise, while maintaining normal habitual food intake during the recording period. Subjects received a login to an online food registration software program (Madlog.dk Aps, Kolding, DK) and received verbal and written information on the usage of the program before day 0. Dietary distribution of macronutrients and intake of total energy was analyzed following the recording period. Supplementary drinks contained 952 kJ in an 8 % solution consisting of either 28 g whey protein + 28 g of carbohydrate for the WPH+CHO supplement or 56 g of carbohydrate for the CHO supplement. Artificially non-caloric flavor was added to the beverages. The carbohydrate source was a standard monosaccharide glucose product. The hydrolysed whey protein (Arla Foods Ingredients Group P/S, Viby J., Denmark) contained 16 % leucine, 6 % isoleucine, 7 % valine, 7 % lysine, 2 % methionine, 5 % phenylalanine, 7 % threonine, 3 % tryptophane, 7 % alanine, 3 % arginine, 6 % aspartic acid, 1 % cysteine, 9 % glutamic acid, 3 % glycine, 2 % histidine, 2 % proline, 7 % serine, and 7 % tyrosine. The percentage of cleaved bonds in the hydrolysed whey protein was 48 %, and the peptide distribution was 21 % <175 kDa (~1 residue), 35 % between 175 and 375 kDa (~2–3 residues), 37 % between 375 and 750 kDa (4–6 residues), and 7 % >750 kDa (>7 residues). The WPH+CHO supplement provided the subjects of the WPH+CHO group an additional 84 g protein per day besides their habitual daily protein consumption.

Measures on indices of muscle damage

This has been previously described by Farup et al. (2014). In brief,

Muscle force: Following 3 min of standardized low intensity cycling on a stationary ergometer cycle (Monark, Varberg, Sweden), the participants were seated in an isokinetic dynamometer (Humac Norm, CSMI, Stoughton, USA) as previously described. Isometric Maximal Voluntary Contraction (MVC) was measured at 70° knee flexion (0° equals full extension). Participants were allowed four trials (however, if a participant continued to improve, additional trials were provided) and all contractions were interspaced by one-minute of recovery. Before each trial, a verbal instruction to contract as "fast and forcefully as possible" was given. Participants were not allowed to use a counter-movement (stretch–shortening cycle movement) before exerting a maximal knee extension. All trials were sampled at 1,500 Hz. The offline analyses were performed in custom-made software (Labview 2011, National Instruments Corporation, TX, USA). MVC was determined as the highest peak torque from the best trial.

Muscle soreness: this was evaluated for both the exercise and the non-exercise control leg in a standardized fashion before the exercise bout and at 24, 48, 72, 96, and 168 h following exercise and was always conducted as the first test. Subjects were asked to rise from a seated chair position and slowly lower back onto the chair using only the leg to be evaluated. Subjects evaluated knee extensor muscle soreness on a Visual Analog Scale (VAS) of 100 mm going from no pain at all (0 mm) to worst possible pain (100 mm), previously described as a method for identification of pain (Bijur et al. 2001). All VAS scores were later analyzed by a blinded investigator.

Creatine kinase: Blood samples were collected before exercise and at 24, 48, 72, 96, and 168 h following exercise. Venous blood samples were collected in 10 mL tubes, cooled for 1 h and centrifuged (1,500*g* for 10 min at 4 °C), and the serum phase was then stored at −80 °C until further analysis. Serum samples were analyzed for creatine kinase content by use of commercial kit applied in a multi-analyser system (Cobas c 311/501, Rotkreuz, Switzerland).

Western blotting

The frozen muscle biopsies (approximately 30 mg) were freeze dried and subsequently homogenized in an ice-cold buffer containing: 20 mMTris, 50 mMNaCl, 250 mM sucrose, 50 mMNaF, 5 mM $Na_4P_2O_7$, 1 % Triton x-100, 2 mM DTT and 1:100 Halt protease inhibitor cocktail (cat # 78,430, Thermo Scientific, IL, USA.). An automated Precellys 24 (bertin technologies, France) bead-based grinder was used for the homogenization. Samples were rotated for 15 min at 4 °C and insoluble materials were centrifuged off at 19,000 × *g* for 20 min at 4 °C. Protein concentration of the supernatant was determined using a Bradford assay (Bio-Rad, CA, USA). Protein concentrations of the samples was equalized with milli-Q water and the samples were denatured by mixing with 4 × Laemmli's buffer (Bio-Rad, CA, USA) and heating at 95 °C for 5 min. Proteins were separated by SDS-PAGE on precast Stain Free 4-15 % gels (Bio-Rad, CA, USA). Proteins were electroblotted onto PVDF membranes (Bio-Rad, CA, USA). Control for equal loading was performed using the stain-free technology (Gurtler et al. 2013). Membranes were blocked for 2 h in 0.1 % I-block TBST solution (Applied Biosystems, CA, USA) and incubated overnight with primary antibodies. The membranes were then incubated 1 h with Horseradish peroxidase-conjugated goat anti-rabbit (cat # 2,054, Santa Cruz, TX, USA) secondary antibody (concentration: 1:2,500 I TBST for all—except for MuRf1 which was 1:5,000). Proteins were visualized by chemiluminescence (Thermo Scientific, MA, USA) and quantified with an UVP imaging system (UVP, CA, USA). Precision Plus Protein All Blue standards was used as markers of molecular weight (Bio-Rad, CA, USA). Membranes were initially incubated with phospho-specific antibodies. Subsequently, the antibodies were stripped with a Restore Plus WB solution (cat # 46,430, Thermo Scientific, IL, USA), and the membranes were reblocked and re-incubated with antibodies raised against epitopes on sites not modified by phosphorylations. Antibodies against mTOR (conc. 1:1,000, cat # 2,972), phospho-specific mTOR (Ser^{2448}) (conc 1:1,000, cat # 2,971), Akt (pan) (conc. 1:1,000, cat # 4,691), phospho-specific Akt (Ser^{473}) (conc. 1:1,000, cat # 9,271), 4E-BP1 (conc. 1:2,000, cat # 9,644), non-phospho-specific 4E-BP1 (Thr^{46}) (conc. 1:000, cat # 4,923), p70S6K (conc. 1:500, cat # 9,202), phospho-specific p70S6K (Thr^{389}) (conc. 1:500, cat # 9,205), rp S6 (conc. 1:1,000, cat # 2,217), phospho-specific rp S6 (Ser 235/236) (conc. 1:2,000, cat # 4,858), FoxO3a (conc. 1:1,000, cat # 2,497), phospho-specific FOXO3a (Ser^{253}) (conc. 1:1,000, cat # 9,466), FOXO1 (conc. 1:1,000, cat #2,880), phosphospecific FOXO1 (Ser^{256}) (conc. 1:1,000, cat #9,461), were purchased from Cell Signalling Technology (Danvers, MA, USA). MuRF1 (conc. 1:1,000, cat # MP3401) was purchased from ECM Biosciences, KY, USA.

Data presentation and statistical analysis

Phosphoprotein data were expressed as a ratio of phosphorylated/expressed protein measured on the same membrane (probing, stripping, and reprobing) for each target, except for phospho-FOXO1, and phospho-FOXO3a, which were normalized to the total amount of protein loaded, from the specific sample, as changes were observed in expressed protein. Total MuRF1 protein was also measured was normalized to the total amount of protein loaded, from the specific sample. The total amount of protein loaded was measured using the Stain Free Technology (Gilda and Gomes 2013; Gurtler et al. 2013).

All protein data were log transformed before statistical analyses to reach normal distribution. The basal pre-exercise levels of phosphorylation of the two supplement groups were compared by *t*-test. No differences between supplement groups were found and the differences from pre-level phosphorylation at 3, 24, and 48 h post-exercise were calculated and analyzed for the effects of time and supplement. Statistical analysis was performed separately for the exercised and the rested leg using a mixed-effect two-way ANOVA with repeated measures for time. The repeated measures on the same subject within time were accounted for in the model by using subject as a random effect. Linear pairwise comparisons were used post-hoc to compare differences within and between the individual conditions. To investigate the association between MVC and phosphorylation levels at 3 h following exercise and supplementation, of measured catabolic and anabolic signaling proteins, a Pearson product-moment correlation coefficient was employed. The level of significance was set at $p < 0.05$. All statistical analysis was performed using Stata (Stata v 12.1, StataCorp LP, College Station, Texas, USA) and graphs were designed in SigmaPlot (SigmaPlot v 11.0, Sysstat Software, Inc. San Jose, California, USA).

Results

Work performed during the experimental trial

Total work performed during exercise was 33.6 [30.5; 36.6] kJ and 31.0 [29.2; 32.9] kJ for the WPH+CHO and CHO-group, respectively. Work performance decreased as the number of exercise sets progressed ($p < 0.01$), with no differences between groups.

Monitoring of habitual dietary intake

The dietary analysis (not including provided supplements) revealed no group difference in total energy intake and macronutrients distribution [protein 18 % (17; 19)],

carbohydrate 51 % (50; 53), fat 31 % (29; 33), energy 8,532.7 (7,869.3; 9,196.0) kJ/day.

Indices of muscle damage

These results have been described in detail previously (Farup et al. 2014). In brief;

Muscle force: MVC decreased by 23 ± 4 and 27 ± 3 % at 24 h following exercise ($p < 0.001$) for WPH+CHO and CHO groups, respectively, with a gradual return towards baseline during the post-exercise recovery time course, and remained depressed compared to baseline at 168 h ($p < 0.001$), with no group differences observed.

Muscle soreness: In the exercise leg, a group × time interaction ($p < 0.05$) was noted. Both groups showed a peak in muscle soreness at 24–48 h ($p < 0.001$) and this remained elevated at 96 h ($p < 0.05$). Complete return to baseline was observed at 168 h for both groups. In the non-exercise control leg, a time effect ($p < 0.001$) was also noted, with a time course similar to the exercise leg and with no differences between dietary supplementation types. At all time points (24–96 h), the magnitude of soreness was greater in the eccentric than the control leg ($p < 0.001$).

Creatine kinase: Pre-level serum CK levels were 135.8 [83.9; 219.9] IU/L and 105.2 [74.4; 148.7] IU/L for WPH+CHO and CHO groups, respectively. At 24 h CK levels were equally elevated to 477.7 [318.7; 716.0] IU/L and 502.5 [318.7; 880] UI/L for WPH+CHO and CHO ($p < 0.001$), respectively, and remained elevated at 48 h before gradually returning towards baseline ($p < 0.001$), with no group × time interaction.

Protein signaling

Representative blots are shown in Fig. 2. Phosphorylation changes during recovery from high intensity eccentric exercise are shown for Akt, and catabolic signaling proteins in Fig. 3, and for anabolic signaling proteins in Fig. 4. All results are presented as fold change from the pre-level.

For p-Akt, a main effect of supplement was observed in the non-exercised leg, with the CHO supplement producing the highest p-Akt levels (Fig. 3a). In the exercise leg (Fig. 3b), a supplement × time interaction was observed; at 3 h post-exercise the p-Akt levels differed for the WPH+CHO and CHO groups, i.e. levels were elevated by 1 ± 25 vs. 91 ± 19 % ($p < 0.05$), compared to basal level, respectively.

For p-FOXO1, the non-exercise leg (Fig. 3c) showed a supplement × time interaction ($p < 0.05$). At 3 and 24 h, the phosphorylation levels were equally decreased in the supplement groups by 54 ± 12 and 62 ± 12 % ($p < 0.001$), respectively, while at 48 h, WPH+CHO, but not CHO, had returned to baseline ($p < 0.01$). In the exercise leg p-FOXO1 (Fig. 3d) showed a main effect of time ($p < 0.001$); at 3, 24, and 48 h the phosphorylation levels was decreased by 23 ± 9, 68 ± 9, and 61 ± 14 %, respectively.

For p-FOXO3a, the non-exercise leg (Fig. 3e) showed main effect of time ($p < 0.001$): at 3, 24, and 48 h the phosphorylation level was decreased by 60 ± 18, 90 ± 15, and 44 ± 23 %, respectively. In the exercise leg p-FOXO3a (Fig. 3f) showed a main effect of time ($p < 0.001$): at 3, 24, and 48 h the phosphorylation level was decreased by 31 ± 14, 60 ± 14, and 42 ± 16 %, respectively.

For MuRF1, no differences with supplement and/or time were observed (Fig. 3g, h).

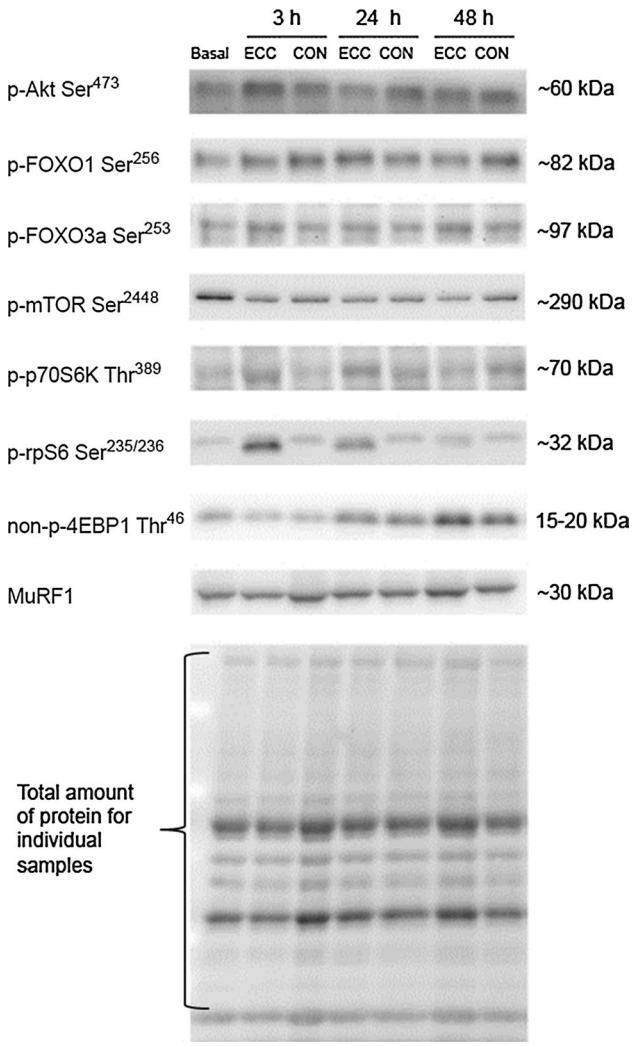

Fig. 2 Representative western blots are shown for phospho-specific Akt downstream catabolic and anabolic signaling proteins MuRF1 protein (*top figure*) as well as total amount of protein for specific samples (*bottom figure*). Blots are shown for the eccentric exercise leg (ECC) and the resting control leg (CON) at pre and 3, 24, and 48 h post single-bout eccentric exercise

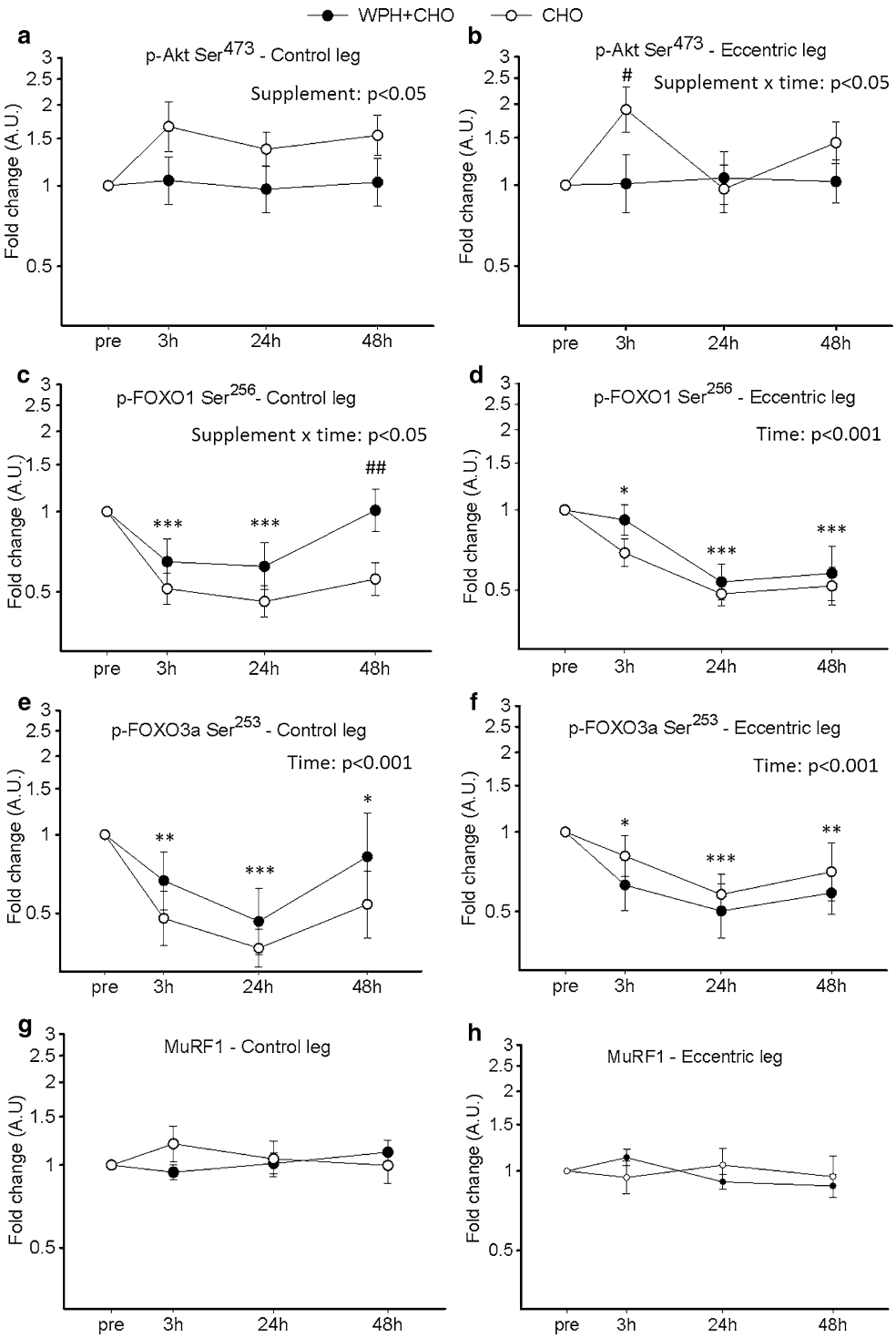

Fig. 3 Akt-FOXO signaling. Phosphorylation changes in the non-exercised control leg and the exercise leg are shown for **a**, **b** Akt kinase (p-Akt), **c**, **d** Forkhead box class O 1 (p-FOXO1) **e**, **f** Forkhead box class O 3a (p-FOXO3a), and **g**, **h** Muscle ring finger1 (MuRF1). The relative fold changes in phosphorylation levels are shown as geometric means ± back-transformed SE at 3, 24, and 48 h of post-exercise recovery compared to pre-level. The changes in the supplement groups (WPH+CHO and CHO) are shown separately for the non-exercised control leg and the eccentrically worked leg. Overall significant ANOVA results are shown in upper right corner of each graph. Differences from pre-level at specific time points are denoted by $^{*}p < 0.05$, $^{**}p < 0.01$ or $^{***}p < 0.001$, respectively. Significant group differences are denoted by $^{\#}p < 0.05$, or $^{\#\#}p < 0.01$

For p-mTOR (Fig. 4a), no effects of supplement or time were observed in the non-exercise control leg. In the exercise leg (Fig. 4b) there was a main effect of time; at 3 h p-mTOR was increased by 50 ± 9 % ($p < 0.05$). For p-p70S6 K the non-exercise control leg (Fig. 4c) showed an effect of time; at 48 h the phosphorylation level was decreased by 55 ± 27 %. In the exercise leg (Fig. 4d) there was a main effect of time; at 3 h p-p70S6 K was increased by 149 ± 27 %. For p-rpS6 the non-exercise control leg (Fig. 4e) showed an effect of time; at 3 h the phosphorylation level was increased by 131 ± 41 %. In the exercise leg (Fig. 4f), there was a main effect of time; at 3 h p-rpS6 was increased by 748 ± 36 %. For non-p-4EBP1 no differences with supplement and/or time were observed (Fig. 4g, h).

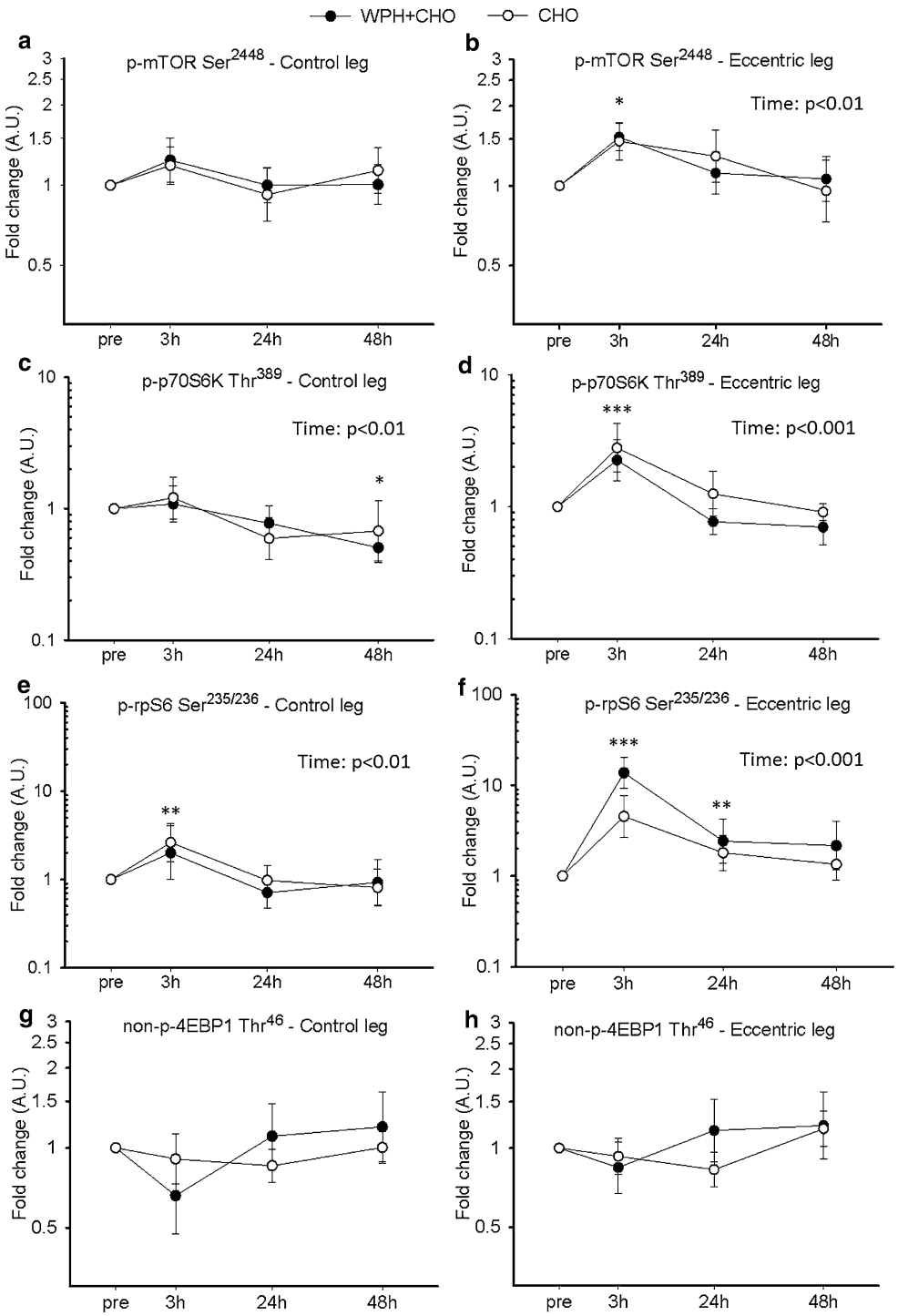

Fig. 4 mTOR signaling. Phosphorylation changes in the non-exercised control leg and the exercise leg are shown for **a**, **b** mammalian target of rapamycin (p-mTOR), **c**, **d** p70S6 kinase (p-p70S6 K), **e**, **f** ribosomal protein S6 (p-rpS6), **g**, **h** (non-phospho) eukaryotic initiation factor 4E binding protein (4E-BP1). The relative fold changes in phosphorylation level are shown as geometric means ± back-transformed SE at 3, 24, and 48 h of post-exercise recovery compared to pre-level. The changes in the supplement groups (WPH+CHO and CHO) are shown separately for the non-exercised control leg and the eccentrically worked leg. Overall significant ANOVA results are shown in upper right corner of each graph. Differences from pre-level at specific time points are denoted by $*p < 0.05$, $**p < 0.01$ or $***p < 0.001$, respectively

To investigate if rate of regain of MVC was associated with phosphorylation levels of the measured catabolic and anabolic signaling proteins at 3 h post-supplement exercise a Pearson product-moment correlation was performed. No association was noted between MVC and any phosphorylation levels of the measured proteins.

Discussion

The aim of the current study was to elucidate how divergent isocaloric supplements would affect signaling for muscle protein turnover during recovery from muscle-damaging eccentric exercise. Main findings comprised; (1) that, despite p-Akt in the ECC leg exhibited an early

increase only with CHO, downstream increases in anabolic signaling and decreases in atrophy signaling, did not differ between supplements, and; (2) that signaling effects were observed in the non-exercise control leg.

The effect of dietary supplementation type on recovery from muscle-damaging exercise

The ability of our ECC protocol to evoke muscle damage was evaluated via post-exercise changes in isometric muscle force loss, plasma muscle creatine kinase and muscle soreness. Separately, the reliability of each of these indices as solid markers of extent of muscle damage are debatable, but collectively patterns and magnitude of response serve to support that muscle damage was indeed inflicted (Brancaccio et al. 2010; Hyldahl and Hubal 2014). Given that muscle force generating capacity during post-exercise recovery from ECC is commonly considered one of the most reliable indirect markers of muscle damage and because muscle force and muscle function are often interrelated (Hyldahl and Hubal 2014), we chose to focus on how the rate of regain of muscle force would be affected by divergent types of dietary supplements. More specifically, given the attention received by protein supplementation rich in BCAA, we aimed to compare a leucin-rich whey protein-based supplement to isocaloric CHO. Moreover, CHO was also added to the protein supplement to mimic commonly applied commercially available supplements. In result of our ECC protocol, a muscle force loss in the order of approximately 25 % was observed 24 h post-exercise which gradually returned towards pre-exercise levels, though still not achieving complete regain at 168 h (published in Farup et al. 2014). This pattern and magnitude of response is in concordance with previous observations (Clarkson and Hubal 2002; Jackman et al. 2010). However, no added effect was observed with WPH+CHO on rate of regain of muscle force generating capacity when compared to isocaloric CHO, whereas a small attenuation in muscle soreness was observed at the 96 h time point after exercise with CHO (published Farup et al. 2014).

This is in opposition to the findings of some, but not other studies on the effects of protein supplementation on recovery from muscle-damaging ECC (Buckley et al. 2010; Cooke et al. 2010; Jackman et al. 2010). Accordingly, in a study by Cooke et al. (2010), attenuated muscle force loss was observed on days 3–7 after ECC with whey protein isolate supplementation (also containing some CHO) compared to isocaloric CHO supplementation. In another study by Buckley et al. (2010), acute muscle force loss was surprisingly more than re-established to above pre-exercise baseline level with whey protein hydrolysate supplementation allready at 6 h after muscle-damaging ECC, whereas whey protein isolate, similar to non-caloric placebo, produced no effect. Adding to complexity, in a study by Jackman et al. (2010), BCAA supplementation did not ameliorate muscle force loss compared to non-caloric placebo supplementation during post-exercise recovery from muscle-damaging ECC, while attenuation of muscle soreness was observed.

Differences in amino acid and/or BCAA composition (i.e., not outlined in the studies by Cooke et al. and Buckley et al.), the time course of measurement and sample size issues all constitute factors that may likely contribute to differences between studies. Due to the invasive procedures of our protocol and our attempt to provide power to the results (i.e., multiple biopsies from 24 subject and a paired design), for practical reasons, we chose to omit a non-caloric control. Thus, we are not able to say if added supplementation even had an added effect on recovery compared to habitual daily intake of macronutrients. However, given the observed rate of recovery (i.e. still not full recovery at 168 h), our results do not differ much from studies on recovery from muscle-damaging ECC, that did not include added dietary supplementation (Paulsen et al. 2007; Vissing et al. 2008). Rather, our results favor the contention that BCAA-rich protein and/or CHO supplements does not improve rate of regain of muscle force and function compared to habitual dietary intake.

The effect of dietary supplementation type on signaling for muscle protein turnover during recovery from muscle-damaging exercise

Among studies that focus on possible advantageous effects of protein/BCAA supplementation during recovery from muscle-damaging ECC, to our knowledge, none of these comprise investigation on myocellular signaling mechanisms potentially involved in regeneration and remodeling. However, studies on exercise involving eccentric contractions demonstrate that such exercise modality can influence both anabolic and atrophy signaling pathways (Lueders et al. 2011; Stefanetti et al. 2014). We therefore wanted to investigate if dietary supplementation could accentuate anabolic signaling and/or attenuate atrophy signaling during post-exercise recovery from muscle-damaging ECC exercise, thereby potentially facilitating myofibrillar restoration and/or influencing rate of muscle force regain.

As for anabolic signaling through mTOR, p70S6K and rpS6, our ECC protocol produced early increases in p-mTOR as well as downstream p-p70S6K and p-rpS6, while no difference was observed for non-phospho-4EPB1. A similar pattern of phosphorylation of p70S6K and rpS6 was seen by Eliasson et al. (2006) following maximal eccentric contractions in the fasted state, although this relied on a protocol designed to evoke muscle accretion, rather than muscle damage. In the current study, for practical reasons, we did

not include measurements of protein synthesis. However, increases in protein synthesis have previously been observed to be associated with increased phosphorylation of mTOR pathway proteins (Cuthbertson et al. 2006). Furthermore, in previous studies of our group, we have observed that traditional as well as eccentrically based resistance exercise conducted by hypertrophy principles can activate the mTOR signaling pathway. Therefore, it can reasonably be assumed that protein synthesis was activated to engage in de novo synthesis of myofibrillar protein as a part of required regeneration or remodeling to better resist muscle damage on re-exposure to similar exercise. However, even though anabolic signaling was increased, it was not observed to differ between supplements. This is in line with our previous findings, where the same supplement types were used to simultaneously evaluate potential differences between isolated concentric versus eccentric exercise by principles to promote muscle accretion rather than muscle damage (Rahbek et al. 2014; Vissing et al. 2013). The lack of difference between supplement types may relate to the fact that our supplements, although of different composition, were isocaloric. This suggests that the accumulated stimulation of mTORC1 and downstream signaling was similar even if partly different signaling paths to mTORC1 activation were stimulated by WPH and CHO. In accordance, with regard to the increase in p-Akt exclusively with CHO supplementation, this indicates that the insulinotrophic effect of CHO superseded that of the WPH+CHO supplement, thus providing a stronger stimulus for anabolic signaling through an Akt-mTORC1-axis. On the other hand, the high content of the amino acid leucine in the WPH+CHO supplement may have exerted greater direct effect on mTORC1 phosphorylation.

As for signaling for protein degradation, FOXO transcription factors are believed to engage in regulation of components of ubiquitin-proteasome system such as the E3-ligases MuRF1 and atrogin-1 (Sandri 2008; Schiaffino et al. 2013). In this regard, Akt is believed to possess a dual role. Accordingly, while stimulating anabolic signaling through mTORC1, Akt is also believed to simultaneously phosphorylate FOXO transcription factor family members such as FOXO1 and FOXO3a, to retain them in the cytosol, thereby inhibiting gene expression of MuRF1 and Atrogin-1 (Sandri 2008; Schiaffino et al. 2013). In the current study, even though Akt was transiently activated only in the CHO group at 3 h post-exercise, phosphorylation of both FOXO1 and FOXO3a was downregulated in both supplement groups and remained downregulated up to 48 h following ECC. Therefore, we propose that other upstream mediators than Akt, may have influenced FOXO phosphorylation. This pattern of decreased phosphorylation of FOXO transcription factors is not unprecedented. Previous results by us demonstrated that isolated eccentric resistance exercise conducted by hypertrophy-inducing principles, produced a similar downregulation in phosphorylation of FOXO1 and FOXO3a independently of whether WPH+CHO or CHO was provided (Stefanetti et al. 2014). On the other hand, other previous studies by us and others strongly suggest that gene expression of atrogin-1 is downregulated and MuRF1 are upregulated during recovery from both traditional and isolated eccentric exercise and independently of whether protein, CHO or non-caloric supplements are provided (Nedergaard et al. 2007; Reitelseder et al. 2014; Stefanetti et al. 2014). Measurements of atrogin-1 and MuRF1 gene expression were not included in the present study. MuRF1 protein measurements were included, but levels were not observed to change with time or exercise, which is in agreement with our previous observations (Stefanetti et al. 2014) as well as observations by Borgenvik et al. (2012) who observed an increase in MuRF1 protein 3 h following conventional resistance exercise in the fasted state and an no increase in the amount of MuRF1 when resistance exercise was combined with BCAA supplementation (Borgenvik et al. 2012).

Signaling in resting control leg

Somewhat unexpected the phosphorylation level of rpS6 level increased while p-FOXO1 and p-FOXO3a decreased in the resting leg. This could be caused by the systemic effects of ergogenic supplements stimulating the PI3K/Akt/mTORC1 or FOXO pathway. Borgenvik et al. (2012) have previously reported that a unilateral bout of resistance exercise increased mTORC1 signaling at 1, and 3 h in a resting leg post-exercise in both BCAA and non-caloric supplemented groups. Furthermore, such remote effects of localized muscle damage have also recently been described in non-human models, but with inducing factors yet to be fully resolved (Rodgers et al. 2014).

In summary, isocaloric WPH+CHO versus CHO supplements were not observed to promote differences in FOXO-mediated or in mTOR-mediated signaling during recovery/regeneration from muscle-damaging eccentric exercise. Furthermore, since no correlations were established between rate of regain of muscle force and signaling for protein turnover, we do not provide evidence that the signaling pathways in question exert strong influence on rate of muscle force regain through muscle regenerative processes. We cannot exclude that other dietary compositions and/or other strategies for timing of intake of supplements may produce different effects. However, the current results support the contention that supplementation with whey protein rich in leucine does not seem to accelerate recovery from muscle-damaging exercise.

Acknowledgments We thank the participants for their participation in the project. Gitte Kaiser Hartvigsen, and Janni Mosgaard Jensen

(Section of Sport Science, Department of Public Health, Aarhus University, Denmark) are thanked for technical assistance.

Conflict of interest The authors declare that they have no conflict of interest.

Ethical standard All participants were informed about the purpose and the risks related to the study and gave written, informed consent to participate. The study was approved by The Central Denmark Region Committees on Health Research Ethics (ref. no. M-20110179) and performed in accordance with the Declaration of Helsinki.

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