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Post exercise carbohydrate–protein supplementation: phosphorylation of muscle proteins involved in glycogen synthesis and protein translation

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Summary. The enzymes Akt, mTOR, $p70^{S6K}$, rpS6, GSK3, and glycogen synthase interact in the control of protein and/or glycogen synthesis in skeletal muscle, and each has been found to respond to exercise and nutrient supplementation. In the present study, we tested the hypothesis that nutrient supplementation post exercise, in the form of a carbohydrate–protein (CHO–PRO) supplement, would alter the phosphorylation state of these enzymes in a manner that should increase muscle protein and glycogen synthesis above that produced by exercise alone. After a 45 min cycling session followed by sprints and again 15 min later, the subjects ($n = 8$) ingested 400 ml of a CHO–PRO drink (7.8% dextrose and 1.8% protein-electrolyte) or a placebo drink, as assigned using a randomized, counter-balanced design with repeated measures. Biopsies of the vastus lateralis were taken before exercise and at 45 min of recovery. At 45 min after supplementation, CHO–PRO treatment yielded greater phosphorylation of Akt (65%), mTOR (86%), rpS6 (85-fold), and GSK3α/β (57%) than pre-exercise levels ($p < 0.05$). Although $p70^{S6k}$ showed an exercise response after 45 min, there were no differences between treatments. Glycogen synthase (GS) phosphorylation was significantly reduced 45 min after exercise for both treatments, but the reduction in phosphorylation was greatest during the CHO–PRO treatment (3-fold decrease; $p < 0.05$), indicating greater activation of GS following supplementation. No difference between treatments was detected prior to exercise for any of the enzymes. These results suggest that a post exercise CHO–PRO supplement alters the phosporylation levels of the enzymes tested in a manner that should accelerate muscle glycogen synthesis and protein initiation during recovery from cycling exercise.

Keywords: Akt – mTOR – $p70^{S6k}$ – rpS6 – Glycogen – Protein synthesis

Introduction

Muscle protein synthesis is stimulated following exercise, with the increase in protein synthesis reported to persist 2 days following a single bout of exercise (Biolo et al., 1995b; Phillips et al., 1997). Muscle protein degradation, however, is also accelerated following exercise (Biolo et al., 1995b). Consequently, despite the improvement in net protein balance, the rate of muscle protein degradation still exceeds the rate of synthesis in the absence of food intake (Phillips et al., 1997).

Protein synthesis is also stimulated by amino acid intake, which does so without affecting muscle protein degradation (Biolo et al., 1997). At rest in normal volunteers, muscle protein synthesis was reported to increase with intravenous infusion of amino acids, whereas muscle protein degradation did not change (Biolo et al., 1997). It remains unclear, however, if the addition of carbohydrate to amino acid intake would further stimulate net protein synthesis. An interactive effect between amino acids and carbohydrate intake would be expected, given the resultant insulin response to carbohydrate. Although insulin is an important anabolic hormone, its mechanism of action on muscle is controversial. Local hyperinsulinemia created by direct infusion of insulin into the femoral artery resulted in increased protein synthesis at rest in the absence of any change in amino acid concentration (Biolo et al., 1995a). Following exercise, the rate of protein degradation was also attenuated with local hyperinsulinemia, but protein synthesis was not further stimulated (Biolo et al., 1999). The failure of insulin alone to stimulate protein synthesis after exercise may be due to an insufficient supply of amino acids. Therefore, the ingestion of protein with carbohydrates after exercise should allow insulin to exert its stimulatory effect on muscle, which would likely manifest as an interactive effect. That is, the combined effects of hyperinsulinemia and exogenous protein would be greater than the sum of their independent effects. Such an additive effect on protein synthesis

was in fact demonstrated by Miller et al. (2003) following resistance training. However, this finding is not universal (Koopman et al., 2007).

Exercise can promote mRNA transcription and synthesis of specific proteins (Kuo et al., 1999a). However, elevated plasma insulin levels are required to fully activate the translation of this mRNA (Kuo et al., 1999b). Insulin likely exerts its effect on translation through the signal transduction pathway containing the serine/threonine protein kinase referred to as the mammalian target of rapamycin (mTOR) via activation of two kinases, PI 3-kinase and Akt (Kimball et al., 2002). Activated mTOR then regulates protein synthesis and translation through activation of p70 ribosomal S6 kinase ($p70^{S6K}$) and inhibition of the binding protein of eukaryotic translation initiation factor eIF4E (4E-BP1) (Brown et al., 1995; Hara et al., 1997). Inhibition of 4E-BP1 allows eIF4E to bind to eIF4G and form the active eIF4F complex, which is essential for ribosomal assembly (Escobar et al., 2005). Activated $p70^{S6K}$ phosphorylates ribosomal protein (rpS6), which leads to enhanced translation of a particular class of mRNAs that encode proteins such as ribosomal proteins, elongation factors, and binding proteins (Kimball and Jefferson, 2004b). Thus, the activation of $p70^{S6K}$ ultimately results in an increase in the capacity of the cell to synthesize protein.

Amino acids can also activate mTOR but the process by which this occurs has not yet been elucidated (Kimball and Jefferson, 2004a), although recent evidence suggest it could occur through the activation of class 3 PI3-kinase, hVps34 (Nobukuni et al., 2005). However, this possibility has to be viewed with caution because the activation of $p70^{S6k}$ by hVps34 has not been demonstrated by an addition of amino acids, but by an inhibition of $p70^{S6k}$ with the removal of amino acids (Byfield et al., 2005). Amino acids can also indirectly phosphorylate 4E-BP1 and $p70^{S6K}$ via a mTOR independent pathway, which results in inhibition of 4E-BP1 and activation of $p70^{S6K}$ (Kimball and Jefferson, 2004a). Phosphorylation of 4E-BP1and $p70^{S6K}$ is associated with the rate of a subset of mRNA translation (Fujita et al., 2007). Thus, it is likely that ingesting protein with carbohydrate would further enhance protein synthesis by increased phosphorylation of these enzymes.

In addition to the potential role in protein synthesis, the co-ingestion of carbohydrate and protein has been found to increase the rate of muscle glycogen storage after exercise, likely due to a greater insulin response and increased activation of glycogen synthase (GS) (Zawadzki et al., 1992; Ivy et al., 2002). Insulin activates GS by phosphorylation of Akt via the PI3-kinase pathway (Srivastava and Pandeym, 1998). Phosphorylated Akt then activates glycogen synthase via inhibition of glycogen synthase kinase $3\alpha/\beta$ ($GSK3\alpha/\beta$), an enzyme that normally inhibits GS (Cross et al., 1995). The $p70^{S6K}$ kinase can also inhibit $GSK3\alpha/\beta$, thus resulting in activation of GS (Armstrong et al., 2001). Therefore, amino acid intake can both increase GS activity, along with translation initiation, by the activation of $p70^{S6K}$ through the mTOR pathway.

While carbohydrate–protein supplementation likely enhances protein synthesis and glycogen synthesis via the mTOR and PI3-kinase pathways, respectively, the phosphorylation state of the enzymes that comprise these pathways have not been directly assessed after the ingestion of carbohydrate–protein supplement following prolonged continuous cycling exercise. Therefore, the purpose of this study was to investigate the effects of prolonged exercise and carbohydrate–protein supplementation post exercise on protein phosphorylation of key enzymes that regulate both skeletal muscle protein synthesis and glycogen storage. Specifically, we investigated the phosphorylation of Akt, mTOR, $p70^{S6K}$, rpS6, $GSK3\alpha/\beta$, and GS, since the phosphorylation state and interactions of these enzymes are highly correlated with the rate of protein synthesis as well as the conversion of glucose to glycogen. It was hypothesized that a carbohydrate–protein supplement will alter the phosphorylation of these key enzymes 45 min post exercise recovery in a manner that should activate protein synthesis and glycogen storage.

Materials and methods

Subjects

Eight well-conditioned males, who were 23.6 ± 1.2 years of age and lived in Austin, Texas, completed the study. The subjects had a maximum oxygen uptake (VO_2 max) of $62.2 \pm 3.2\,ml \cdot kg^{-1} \cdot min^{-1}$, and weighed $72.5\,kg \pm 3.3\,kg$. Prior to any testing, the testing procedures were explained to the subjects, and a medical questionnaire was then administered to screen subjects for medical conditions that may affect the study such as family history of diabetes, hypertension, and metabolic syndrome. The subjects then completed a consent to participate form, according to the protocol described in The University of Texas at Austin's ''Institutional Review Board Procedures Manual for Faculty, Staff and Student Researchers with Human Participants''. Each subject completed the study in approximately four weeks.

Pre-testing

The subjects reported to the laboratory for a VO_2 max cycling test prior to the experimental trials. First, they were familiarized with the test. After adjusting the seat, the subjects mounted an electrically braked Lode Excalibur Sport bicycle (Lode BV, Groningen, The Netherlands). The subjects wore a heart rate monitor around their chest, and heart rate was

monitored throughout the test (UNIQ Heartwatch model 8799, Computer Instruments, Hempstead, NY). The subjects breathed through a Daniel's valve and respiratory gas samples were measured throughout using a computer-based open-circuit gas analysis system (Max-1 PhysioDyne Instruments Corporation, Quogue, NY). The subjects cycled at 150 watts for the first 4 min. The watts were increased by 25 watts every 2 min until respiratory exchange ratio (RER) was greater than 1.1 and an increase in VO_2 was less than $0.2\,L \cdot min^{-1}$. After completion of testing, the subjects were provided instructions regarding dietary intake and physical activity prior to testing. The subjects were required to perform standardized workouts on the 2 days before each trial. Diets were controlled during the days preceding each trial. During this period, the subjects ate a normal mixed diet (50% carbohydrate, 35% fat, and 15% protein) with the total caloric content based on dietary recalls recorded during the week prior to the start of testing. The night before each test, a standard liquid meal (Ensure, Abbott Laboratories) was provided. This was followed by a 12 h fast to standardize initial muscle and liver glycogen concentrations.

Experimental trials

On two separate occasions scheduled a minimum of 7 days apart, the subjects reported to the laboratory. The testing consisted of three parts: pre-exercise, exercise, and post-exercise.

In the pre-exercise phase, the subject was weighed and a heart rate monitor was secured around the subject's chest. Next, a 20-gauge catheter was inserted into a large forearm vein. The catheter was flushed with sterile saline at regular intervals during exercise and following each blood draw to prevent blockage in the catheter. Once the catheter was in place, the thigh was cleansed with 10% Betadine solution, and then 1.8 ml of a local anesthesia (1% Lidocaine Hydrochloride Injection, Elkins-Sinn, Inc., Cherry Hill, NJ) was used to prepare the leg for the muscle biopsy. A 5–8 mm incision was made through the skin and fascia, 7 cm from the midline of the thigh on the lateral side and 14 cm above the patella. Once the bleeding had stopped, the muscle biopsy was taken and pressure was then reapplied to the incision to stop bleeding. The biopsy was then trimmed of adipose and connective tissue and frozen in liquid nitrogen at −80 °C for subsequent analysis. Once bleeding had stopped, the incision was closed with an adhesive strip and a pressure pack was affixed over the incision. The subject then mounted an electrically braked cycle ergometer (Lode Excalibur) equipped with cycling pedals and remained seated quietly for approximately 2 min. A resting blood sample (5 ml) was taken and resting heart rate recorded.

In the exercise phase, the subject started with a warm-up cycling at 50% of VO_2 max for the first 3 min of exercise. The intensity was then increased to 75% of VO_2 max for the next 45 min. The subject then performed 5 one-minute sprints at 90% of VO_2 max with 1-min of cycling at 45% VO_2 max between sprints. Watts to elicit the appropriate percentage of VO_2 max were estimated using the following regression formula: VO_2 (ml/min) = (Watts × 12.5) + 300. Respiratory gas samples (VO_2, VCO_2) were measured with a computer-based open-circuit gas analysis system (Max-1 PhysioDyne Instruments Corporation, Quogue, NY) during the 5–10 min and 40–45 min of exercise to ensure the subjects were cycling at the appropriate intensity. Heart rate was monitored throughout each trial. A blood sample (5 ml) was taken after 45 min of exercise and at completion of exercise.

Upon completion of the exercise phase, (recovery phase) and 15 min post-exercise, the subjects immediately ingested 400 ml of either a flavored aspartame sweetened placebo drink containing appropriate electrolytes (placebo), or a 7.8% dextrose/1.8% protein-electrolyte (w/v) supplement containing the same electrolytes as the placebo (CHO–PRO). The protein was a whey isolate and the electrolytes were sodium, potassium and magnesium in concentrations of 53, 18, and 21 mg/100, respectively. After a 45 min recovery period, the second biopsy was taken from the leg and the pressure pack was reapplied. Biopsies altered between legs for each treatment (e.g., right leg for treatment 1 and left leg for treatment 2). Blood samples (5 ml) were drawn at 15 and 45 min of recovery.

Blood analysis

Blood samples (5 ml) were analyzed for glucose, insulin, and lactate. The blood samples were transferred to tubes containing ethylenediaminetetraacetic acid (EDTA) solution (24 mg/ml, pH 7.4). One milliliter of each sample was transferred to tubes containing 2 ml of 10% perchloric acid (PCA). Collection tubes were kept on ice during the trial and centrifuged at 1000 *g* for 10 min at 4 °C upon collection of the last blood sample. Plasma and PCA extracts were transferred to 12 × 75 mm plastic test tubes and immediately frozen at −80 °C for later analysis.

Blood glucose was measured immediately after collection using One Touch Basic glucose analyzer (LifeScan Inc., Milipitas, CA). The analyzer was calibrated, and values were checked against a YSI 23A glucose analyzer (Yellow Springs Instrument, Yellow Springs, OH). Enzymatic analysis was used to measure lactate (Hohorst, 1965), and radioimmunoassay was used to measure plasma insulin (MP Biomedicals, LLC, Orangeburg, NY).

Muscle tissue preparation and analysis

Muscle samples (∼40 mg) were weighed and homogenized in ice-cold buffer, containing 20 mM Hepes, 2 mM EGTA, 50 mM sodium fluoride, 100 mM potassium chloride, 0.2 mM EDTA, 50 mM glycerophosphate, 1 mM DTT, 0.1 mM PMST, 1 mM benzamidine, and 0.5 mM sodium vanadate (pH 7.4). Homogenization was performed on ice using Caframo RZR1 Stirrer (Caframo Limited, Wiarton, Ontario, Canada). The homogenate was centrifuge at 14,000 *g* for 10 min at 4 °C and the supernate was then aliquoted to several test tubes and stored at −80 °C for later analysis.

A modified version of the Lowry assay (Lowry et al., 1951) was used to determine protein concentration. Protein phosphorylation was determined using western blotting as previously described by Sakamoto et al. (2004) for Akt, Bolster et al. (2002, 2003) for mTOR, $p70^{S6k}$, and rpS6, and by Tobwin et al. (1979) for GSK3α/β and GS.

Equal amounts of muscle proteins (40 μg for GS, 50 μg for GSK3α/β, mTOR, $p70^{S6k}$, and rpS6, and 80 μg for Akt) were separated by gel electrophoresis, using a sodium dodecyl sulphate (SDS)-Page, 7.5% resolving gel for mTOR and $p70^{S6k}$ and 12% resolving gel for Akt, rpS6, GSK3α/β, and GS. The proteins were then transferred to Polyvinylidene fluoride (PVDF) membranes and blotted in freshly prepared TBS containing 5% nonfat dry milk and 0.06% tris-buffered saline tween-20 (TBST-MLK) for 1 h at room temperature with agitation. The PVDF membranes were then incubated with a primary antibody over night with gentle agitation at 4 °C. Akt was incubated with a rabbit anti-phospho-Akt1/PKBα (Ser473) antibody (1:3000 dilution; Upstate Technology, Lake Placid, NY). Antibodies for mTOR (Ser2448), GS (Ser641), and rpS6 (Ser235/236) were obtained from Cell Signaling (1:1000 dilution; Cell Signaling Technology Inc., Beverly, MA). $p70^{S6k}$ was incubated with an anti-phospho-$p70^{S6k}$ (Thr412) at a 1:2000 dilution (Upstate Technology). After the membranes were washed in 0.06% tris-buffered saline (TBS) solution, they were incubated with a secondary reagent for 2 h at room temperature with agitation. An anti-rabbit IgC HRP-linked antibody (Cell Signaling Technology) was used at a dilution of 1:3000 for Akt and at a dilution of 1:2000 for mTOR, GS, and rpS6. An anti-rabbit IgC HRP-linked antibody was obtained from Upstate Technology for $p70^{S6k}$ (1:4000 dilution). The PVDF membranes were then washed in 0.06% TBS solution, and the antibody-bound proteins were visualized by means of Western Lightning Chemiluminescence Reagent Plus (Perkin Elmer LAS, INC., Boston, MA) according to manufacturer's protocol. Images were scanned using Adobe Photoshop and quantified using Scion Image (Scion Corporation, Frederick, MD). A molecular weight ladder (Bio Rad, Precision Plus protein standard) and a rodent internal loading control were also included on each gel. Equal amounts of protein transferred were confirmed by Coomassie Blue staining.

Table 1. Blood glucose, insulin, and lactate concentrations before, during and following exercise for the carbohydrate–protein (CHO–PRO) and placebo trials

	Time				
	Pre Ex	45 min	Post Ex	15 min Post	45 min Post
Glucose mM					
CHO–PRO	3.72 ± 0.01	3.68 ± 0.27	3.81 ± 0.35	4.55 ± 0.37*	4.06 ± 0.45*
Placebo	3.78 ± 0.15	3.81 ± 0.28	3.85 ± 0.31	3.78 ± 0.23	3.25 ± 0.12
Insulin pM					
CHO–PRO	91.2 ± 9.0	56.9 ± 5.8	67.1 ± 4.7	161.2 ± 26.4	503.6 ± 106.4*
Placebo	85.0 ± 10.5	52.0 ± 6.6	66.9 ± 7.1	86.6 ± 7.2	76.2 ± 7.0
Lactate mM					
CHO–PRO	1.02 ± 0.36	3.03 ± 0.33	5.55 ± 0.63	2.72 ± 0.33	1.72 ± 0.18
Placebo	0.96 ± 0.14	3.70 ± 1.03	5.67 ± 1.03	2.86 ± 0.50	1.40 ± 0.18

Values are means ± SEM. *Pre Ex* prior to exercise; *Post Ex* post exercise. * Indicates significant difference between treatments ($p < 0.05$)

Research design and statistical analyses

The research design was a randomized, counterbalanced design with subjects receiving both treatments. Two-way analysis of variance for repeated measures (treatment × time) was used to analyze the dependent variables. Significant different differences between means were determined using Fisher's Least Significance Difference post hoc analysis. Differences were considered significant at p-value <0.05. The data were expressed as means ± standard error of the mean (SEM).

Results

There was no difference in oxygen uptake (VO_2), respiratory exchange ratio (RER), carbohydrate oxidation, fat oxidation or perceived effort among treatments during exercise (data not shown). Mean VO_2 did not change during the first 45 min of exercise, ranging between 73.39 ± 1.32 to 73.50 ± 1.01% of VO_2 max ($p > 0.05$). During this time, RER declined significantly during exercise as oxidation of carbohydrates decreased while oxidation of fats increased ($p < 0.05$).

The blood data are presented in Table 1. Blood lactate did not differ between treatments ($p > 0.05$), suggesting that exercise was of equal intensity for both treatments. Blood glucose was also not different between treatments at the onset of exercise or immediately post-exercise ($p > 0.05$). The mean blood glucose at the end of exercise was similar for both treatments. During the post-exercise recovery period, however, blood glucose for the CHO–PRO treatment was significantly greater than for the placebo treatment at 15 and 45 min ($p < 0.05$).

Plasma insulin levels did not differ between treatments prior to exercise, during exercise, and following the first 15 min of recovery. However, at 45 min of recovery, plasma insulin level for the CHO–PRO treatment was 5-folds greater than for the placebo treatment ($p < 0.05$) (Table 1).

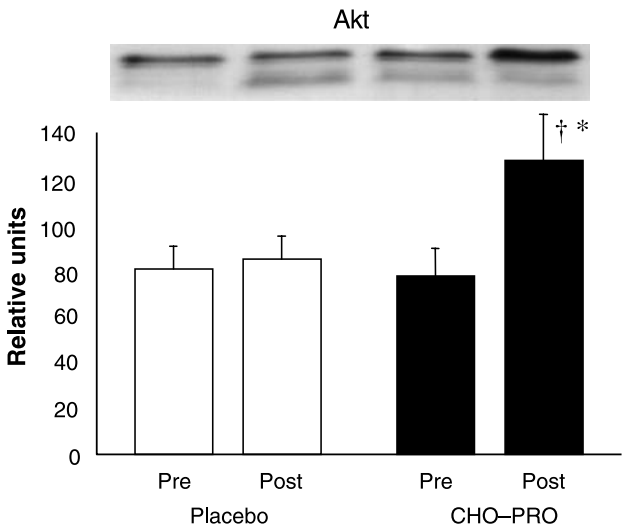

Fig. 1. Phosphorylated Akt (% of standard) before exercise for placebo treatment and carbohydrate–protein (CHO–PRO) treatment and at 45 min of recovery for placebo treatment and CHO–PRO treatment. †Significantly different from corresponding pre-exercise value. *Significantly different than post-exercise placebo treatment

Phosphorylation of Akt was similar for both treatments prior to exercise. However, at 45 min of recovery, phosphorylation of Akt was significantly increased by 65% following the CHO–PRO treatment, but not increased following the placebo treatment (Fig. 1).

The phosphorylation of mTOR followed a similar pattern as Akt (Fig. 2). No difference was detected in the phosphorylation state of mTOR prior to exercise between

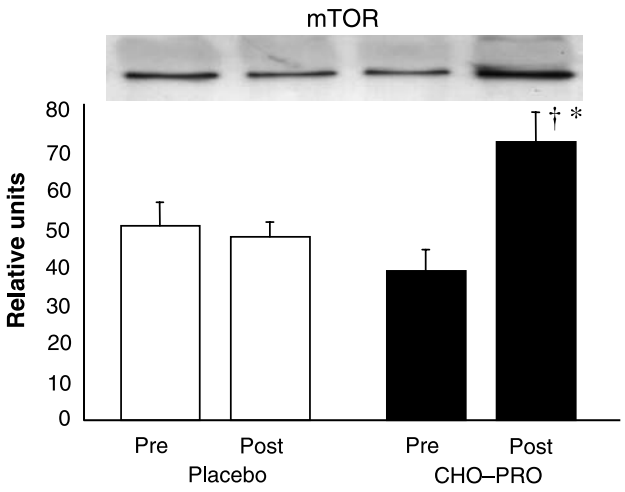

Fig. 2. Phosphorylated mTOR (% of standard) before exercise for placebo treatment and carbohydrate–protein (CHO–PRO) treatment and at 45 min of recovery for placebo treatment and CHO–PRO treatment. †Significantly different from corresponding pre-exercise value. *Significantly different than post-exercise placebo treatment

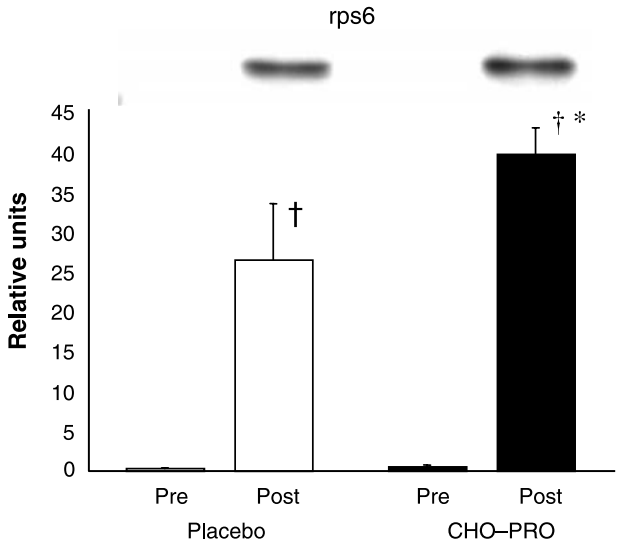

Fig. 4. Phosphorylated rpS6 (% of standard) before exercise for placebo treatment and carbohydrate–protein (CHO–PRO) treatment and at 45 min of recovery for placebo treatment and CHO–PRO treatment. †Significantly different from corresponding pre-exercise value. *Significantly different than post-exercise placebo treatment

the CHO–PRO and placebo treatment. However, at 45 min of recovery, phosphorylation of mTOR increased significantly by 86% with the CHO–PRO treatment ($p < 0.05$) but did not increase with the placebo treatment.

The phosphorylation of $p70^{S6k}$, a downstream target of mTOR, was significantly above pre-exercise levels at 45 min post-exercise for both treatments (Fig. 3). Moreover, at 45 min post-exercise the phosphorylation of $p70^{S6k}$ during the CHO–PRO and placebo treatments did not differ in response.

Prior to exercise, phosphorylation of rpS6, a downstream target of $p70^{S6k}$, was minimal for both treatments (Fig. 4). At 45 min of recovery, phosphorylation of rpS6 was greater than pre-exercise levels for both treatments. The increase was greater following the CHO–PRO treatment compared to the placebo treatment, however ($p < 0.05$).

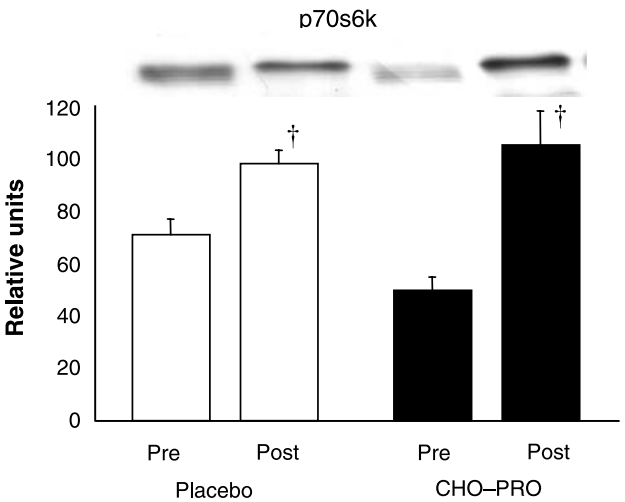

Fig. 3. Phosphorylated $p70^{S6k}$ (% of standard) before exercise for placebo treatment and carbohydrate–protein (CHO–PRO) treatment and at 45 min of recovery for placebo treatment and CHO–PRO treatment. †Significantly different from corresponding pre-exercise levels

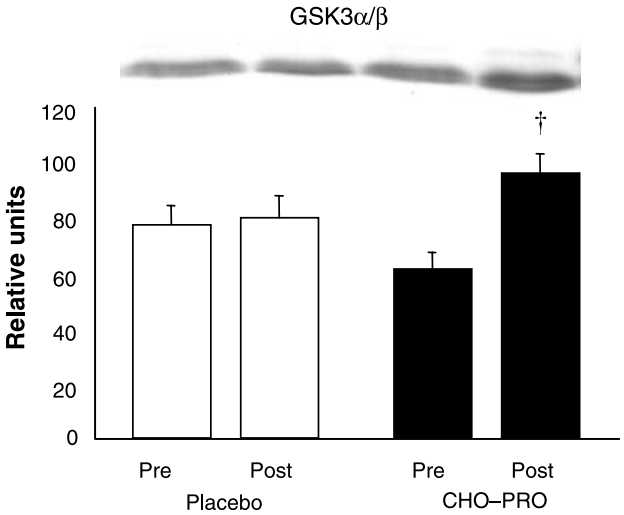

Fig. 5. Phosphorylated GSK3 (% of standard) before exercise for placebo treatment and carbohydrate–protein (CHO–PRO) treatment and at 45 min of recovery for placebo treatment and CHO–PRO treatment. †Significantly different from post-exercise placebo treatment

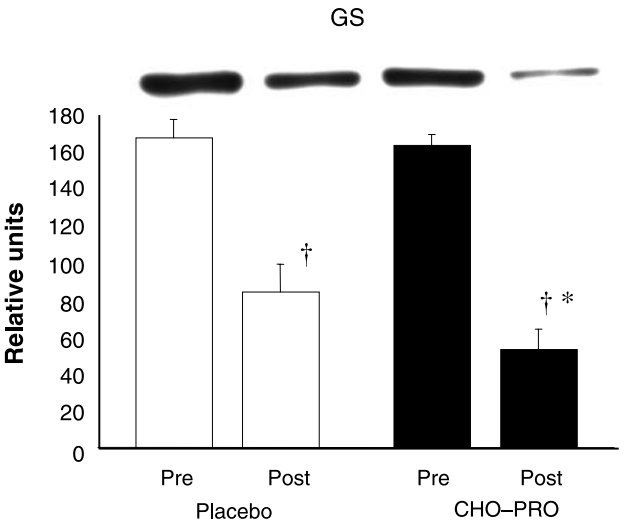

Fig. 6. Phosphorylated GS (% of standard) before exercise for placebo treatment and carbohydrate–protein (CHO–PRO) treatment and at 45 min of recovery for placebo treatment and CHO–PRO treatment. †Significantly different from corresponding pre-exercise value. *Significantly different than post-exercise placebo treatment

GSK3α/β phosphorylation was the same prior to exercise for both treatments. However, at 45 min of recovery, its phosphorylation state was only increased with the CHO–PRO treatment (57% increase above pre-exercise levels; $p < 0.05$), similar to Akt and mTOR (Fig. 5).

GS was highly phosphorylated prior to exercise for both treatments. At 45 min of recovery, the phosphorylation state of GS was significantly decreased 3-fold in the CHO–PRO and 2-fold in the placebo treatments, with a greater decrease observed in the CHO–PRO treatment ($p < 0.05$) (Fig. 6).

Discussion

The present study was pursued in an attempt to characterize both the roles of variable intensity endurance cycling exercise and nutrition on the phosphorylation activation of intramuscular signaling pathways involved in muscle glycogen storage and protein synthesis during the post-exercise recovery phase. There have been various studies investigating both the rates of whole-body protein synthesis, and the activation of mTOR signaling events in the post resistance-exercise recovery state. However, to our knowledge, our results provide the first glimpse at the activation of Akt/mTOR signaling events in response to both variable intensity endurance cycling exercise and nutrition supplementation.

The major finding of this study was that supplementation with a CHO–PRO drink after endurance exercise yielded a significantly greater phosphorylation of Akt, mTOR, rpS6, and GSK3α/β after 45 min of recovery compared to placebo. These differences can be directly linked to the nutritional effect of the CHO–PRO supplement since these increases were significant when compared to exercised placebo control at 45 min.

Since the CHO–PRO drink contained only carbohydrates in the form of dextrose, and amino acids in the form of a whey isolate, the effect of the drink on intracellular signaling can be attributed to either greater amino acid availability, increased plasma insulin concentrations, or a combination of the two. The release of amino acids into the blood from whey protein ingestion occurs quickly, and can be closely related to studies demonstrating a link between amino acid infusion and greater rates of amino acid transport into the skeletal muscle (Bos et al., 2003).

Although net muscle protein accretion was not directly measured in this study, amino acid infusion has been previously found to stimulate muscle protein synthesis both at rest (Biolo et al., 1997), and post-resistance exercise (Tipton et al., 1999; Borsheim et al., 2002). In particular, the branched chain amino acid leucine appears to be most effective in promoting positive net muscle protein synthesis (Tipton et al., 1999; Borsheim et al., 2002; Koopman et al., 2005), and stimulating the mTOR pathway at the level of translation initiation (Kimball and Jefferson, 2004b).

The role and relative contribution of cytosolic amino acids, especially BCAAs such as leucine, on activating intracellular signaling is not clear, however, there is evidence that cytosolic amino acids may signal to mTOR through various mechanisms, including the inhibition of tuberin (Gao et al., 2002), and the activation of Ras homolog enriched in brain (Rheb) (Zhang et al., 2003). Another more recently proposed model involves a class III PI3K pathway, independent of tuberous sclerosis 1 and 2 (TSC1/TSC2) (Um et al., 2006).

While previous studies have found the intake of BCAAs to increase mTOR phosphorylation through various models, amino acids appear to have no effect on PKB/Akt, or GSK3α/β phosphorylation in vivo (Hara et al., 1998; Liu et al., 2004). Therefore, in this study, the stimulatory effect of the CHO–PRO drink on increased phosphorylation states of Akt and GSK3α/β with supplementation can be attributed to the increased plasma insulin concentrations, where insulin signaling up-regulated the PI3-kinase cascade. In this pathway, the activation of Akt leads to both the activation of mTOR, and the phosphorylation of GSK3α/β, which in turn inactivates

the kinase activity of GSK3α/β, leading to the activation of GS (Cross et al., 1995; Yeaman et al., 2001). GS is a highly regulated enzyme with its activity controlled allosterically as well by its phosphorylation state, where the activation of the enzyme occurs upon its dephosphorylation. An intracellular increase in glucose-6-phosphate or decrease in glycogen concentration will increase GS activity.

The activation of the PI3-kinase pathway was confirmed in the present study by the observed increase in Akt phosphorylation and reduction in GS phosphorylation after supplementation. GS activation was also increased with exercise, a result that can be directly attributed to the endurance exercise protocol reducing muscle glycogen stores, since there generally exists an inverse relationship between muscle glycogen stores and GS activation (Nielsen and Richter, 2003).

The present study did not demonstrate an exercise effect on mTOR phosphorylation, although there may have been a response that had been attenuated by 45 min post-exercise. Evidence for such an attenuated response has been demonstrated by Mascher et al. (2007), who have found increases in the phosphorylation of mTOR from pre-exercise to 30 min post exercise, but no differences when pre-exercise values were compared to one-hour post-exercise values. Interestingly, although mTOR was phosphorylated with supplementation, there was no additive effect of nutrition on the phosphorylation of its downstream target $p70^{S6K}$ that was found with exercise. Recent studies have shown $p70^{S6K}$ to be strikingly sensitive to nutritional stimuli (Hara et al., 1998; Cuthbertson et al., 2005; Anthony et al., 2007), especially BCAA (Karlsson et al., 2004; Bromstrand et al., 2006). Although an independent nutrient effect for $p70^{S6K}$ phosphorylation was not observed in the present study, $p70^{S6K}$ was both phosphorylated and activated, as demonstrated by an increased phosphorylation of its downstream target rpS6 linked directly to the effect of the CHO–PRO supplement. Any effect of nutrient supplementation on $p70^{S6K}$ phosphorylation may have been masked by the exercise effect found in the placebo group.

The variable intensity endurance cycling exercise stimulus used in the present study had a significant effect on intracellular signaling by increasing the phosphorylation state of rpS6 and $p70^{S6K}$, two enzymes that lie downstream of mTOR in the protein translation pathway. The effect of exercise on $p70^{S6K}$ activation has yet to be fully elucidated. Blomstrand et al. (2006) found no increase in $p70^{S6K}$ phosphorylation at thr-389 after 60 min of endurance exercise. Nor was an increase found 30 min or 60 min after exercise in a more recent study by Mascher et al. (2007). However, Dreyer et al. (2006) was able to find increases in $p70^{S6K}$ two-hours after exercise. Other studies have demonstrated that maximal eccentric contractions activate $p70^{S6K}$ up to two-hours post-exercise (Eliasson et al., 2006), and that the activation of $p70^{S6K}$ with electrical stimulation may be fiber-type specific (Nader and Esser, 2001; Koopman et al., 2006).

Furthermore, rpS6 was found to be further activated by the exercise protocol than with supplementation alone. Along with being activated by hormones and nutrients through the mTOR pathway, rpS6 is also activated by the p38 MAP kinase pathway through ERK1/2 (Roux and Blenis, 2004). rpS6 is directly phosphorylated at ser-235/236 in an mTOR-independent manner by $p90^{RSK}$ (Pende et al., 2004), a component of the ERK1/2 cascade. Williamson et al. (2006) recently demonstrated that 30 min of endurance exercise in rats stimulated both $p90^{RSK}$ and rpS6. The model of rpS6 phosphorylation by $p90^{RSK}$ may help explain the additive positive effect of exercise and supplementation on rpS6 ser-235/236 phosphorylation states observed in the present study.

Taken together, the results of the current study support the role of both carbohydrate and protein in a post-exercise supplement in activating enzymes associated with both glycogen storage and muscle protein synthesis. Carbohydrate via insulin secretion, and protein supplementation in the post-exercise state appears to have two major effects. First, nutrients provided the substrate for both metabolic processes. Secondly, it significantly altered the phosphorylation state of proteins controlling both glycogen and protein synthesis, suggesting an increase in the activity of key enzymes controlling these metabolic processes. Thus our results appear to explain, in part, the previous observations that post-exercise supplementation with carbohydrates and protein will function to increase muscle glycogen recovery and muscle protein synthesis substantially better than when these macronutrients are provided alone (Miller et al., 2003; Anthony et al., 2007). However, our results may differ using untrained, or strength trained individuals (Coffey et al., 2006). Much work remains to be done on characterizing the individual roles of insulin and amino acids on muscle protein synthesis after aerobic cycling exercise.

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