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ORIGINAL ARTICLE

L-Alanylglutamine inhibits signaling proteins that activate protein degradation, but does not affect proteins that activate protein synthesis after an acute resistance exercise

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Abstract Sustamine™ (SUS) is a dipeptide composed of alanine and glutamine (AlaGln). Glutamine has been suggested to increase muscle protein accretion; however, the underlying molecular mechanisms of glutamine on muscle protein metabolism following resistance exercise have not been fully addressed. In the present study, 2-month-old rats climbed a ladder 10 times with a weight equal to 75 % of their body mass attached at the tail. Rats were then orally administered one of four solutions: placebo (PLA-glycine = 0.52 g/kg), whey protein (WP = 0.4 g/kg), low dose of SUS (LSUS = 0.1 g/kg), or high dose of SUS (HSUS = 0.5 g/kg). An additional group of sedentary (SED) rats was intubated with glycine (0.52 g/kg) at the same time as the ladder-climbing rats. Blood samples were collected immediately after exercise and at either 20 or 40 min after recovery. The flexor hallucis longus (FHL), a muscle used for climbing, was excised at 20 or 40 min post exercise and analyzed for proteins regulating protein synthesis and degradation. All supplements elevated the phosphorylation of FOXO3A above SED at 20 min post exercise, but only the SUS supplements significantly reduced the phosphorylation of AMPK and NF-kB p65. SUS supplements had no effect on mTOR signaling, but WP supplementation yielded a greater phosphorylation of mTOR, p70S6k, and rpS6 compared with PLA at 20 min post exercise. However, by 40 min post exercise, phosphorylation of mTOR and rpS6 in PLA had risen to levels not different than WP. These results suggest that SUS blocks the activation of intracellular signals for MPB, whereas WP accelerates mRNA translation.

Keywords Resistance exercise · Whey protein · L-alanylglutamine · Muscle protein breakdown · Muscle protein synthesis · Mammalian target of rapamycin

Abbreviations

Akt	Protein kinase B
AMPK	5′ Adenosine monophosphate-activated protein kinase
FOXO3A	Forkhead box O-3A
IGF-1	Insulin-like growth factor-1
mTOR	Mammalian target of rapamycin
NF-kB p65	Nuclear factor kappa-light-chain-enhancer of activated B cells-p65
p70S6k	70-kDa ribosomal S6 protein kinase
rpS6	Ribosomal protein S6
MPS	Muscle protein synthesis
MPB	Muscle protein breakdown

Introduction

Glutamine is the most abundant amino acid in plasma (550–750 μM) and skeletal muscle (20 mmol/kg wet weight muscle) in humans (Felig 1975; Gleeson 2008). Under normal conditions, the demand for glutamine can be met by synthesis within the skeletal muscle and from dietary proteins (Newsholme and Parry-Billings 1990; Newsholme et al. 2003). Under catabolic conditions such

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as severe illness, trauma, and overtraining, glutamine concentrations in plasma and skeletal muscle may fall below normal levels (Lambertucci et al. 2012; Novak et al. 2002). When the requirement for glutamine exceeds its de novo synthesis, exogenous glutamine intake becomes necessary. Thus, glutamine is classified as a conditionally essential amino acid (Lacey and Wilmore 1990). It was found that the intramuscular glutamine concentration was highly associated with the rate of muscle protein synthesis (MPS) in isolated perfused muscle (MacLennan et al. 1987). Therefore, maintaining or increasing glutamine concentration in skeletal muscle appears crucial for MPS, particularly under a catabolic state. In the past two decades, glutamine supplementation has been used by athletes because it has been reported to increase glycogen synthesis (Bowtell et al. 1999) and protein synthesis (Jepson et al. 1988; MacLennan et al. 1987), and prevent muscle atrophy (Salehian et al. 2006). A dipeptide containing glutamine also was reported to improve cycling exercise performance by increasing time to exhaustion (Hoffman et al. 2010).

MPS is primarily controlled by the phosphorylation of proteins in the Mammalian target of rapamycin (mTOR) signaling pathway. mTOR is an important kinase that integrates upstream signals from muscle contraction, amino acids, and growth factors to stimulate MPS via mediating mRNA translation initiation. Once mTOR is activated by phosphorylation at its Ser2448 site, it further phosphorylates 70-kDa ribosomal S6 protein kinase (p70S6k) (Fingar and Blenis 2004; Fingar et al. 2004). p70S6k activates ribosomal protein S6 (rpS6), which results in translation of mRNA to increase capacity of protein synthesis (Jastrzebski et al. 2007). Conversely, the main suppressors limiting muscle protein breakdown (MPB) are the muscle ubiquitin E3 ligases. Expression of these ligases is regulated via the forkhead box O-3A (FOXO3A) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB)-dependent signaling pathways (Cai et al. 2004; Nakashima and Yakabe 2007; Stitt et al. 2004). Protein intake can elicit MPS via the activation of the mTOR signaling pathway at rest or after exercise (Atherton et al. 2010; Moore et al. 2009). However, there is limited information on the effect of glutamine on signaling pathways that control protein synthesis and degradation. A recent research study suggested that glutamine alleviates the loss of muscle mass in diabetic rats via the activation of the mTOR signaling pathway as well as the inhibition of ubiquitin E3 ligases expression (Lambertucci et al. 2012). However, it remains unclear whether glutamine mediates these signaling pathways in conditions of resistance exercise.

As a nutritional supplement, L-Gln is usually provided in the form of a capsule or as a powder. It can also be supplied in liquid form, but requires the liquid be of low pH to enhance palatability and reduce microbial growth. However, due to low solubility and stability of glutamine in low pH solutions (Arii et al. 1999; Furst 2001), glutamine dipeptides such as L-alanylglutamine (AlaGln) have been designed to improve upon these physical limitations in order for glutamine to be used in sports drinks and health products (Harris et al. 2012; Rogero et al. 2006). Compared with glutamine or wheat protein, AlaGln facilitates glutamine absorption and increases plasma glutamine concentration to a higher level (Harris et al. 2012; Rogero et al. 2006). AlaGln administration has also been found to attenuate muscle damage as suggested by lower inflammation biomarkers following prolonged endurance exercise (Cruzat et al. 2010). In the present study, we investigated the effects of acute Sustamine™ (SUS) supplementation, a dipeptide composed of alanine and glutamine, on the signaling pathways controlling MPS and MPB post resistance exercise. Comparisons were made relative to whey protein (WP) supplementation.

Materials and methods

Animals

A total of eighty-nine male Sprague–Dawley rats were obtained at approximately 2–3 months of age from Charles River (Wilmington, MA). Rats were housed two per cage and provided standard laboratory chow (Prolab RMH 1800 5LL2, LabDiet, Brentwood, MO) and water ad libitum. The temperature of the animal room was maintained at 21 °C. A reverse artificial 12-h dark–light cycle was set with the light phase from 8:00 pm to 8:00 am. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Texas at Austin and conform to the guidelines for the use of laboratory animals published by the United States Department of Health and Human Resources.

Exercise familiarization

Following 1 week of acclimation to their new environment, each rat underwent three repeated sessions of ladder climbing separated by 1 day between each session to familiarize them with the exercise protocol. During the familiarization period, the rats carried no weights. The rats climbed a ladder 1 m in height on an incline of 85° with 2 cm grid steps eight times each practice session with a 2-min rest between climbs. The rats also completed three practice sessions of climbing separated by 1 day between each session with 50, 60, and 70 % of their body mass attached to their tails, respectively. The weight was attached at the base of the tail with foam tape (3 M Conan) and a Velcro strap. Rats were encouraged to climb by lightly tapping their tails with a bottlebrush.

Experimental protocol

On the experimental day, following a 2-h fast, rats climbed the ladder 10 times with a weight equal to 75 % of their body mass attached at the base of the tail. There was a 2-min rest period between each climb. Once the exercise protocol was completed, a 0.7 ml blood sample was collected from the tip of the tail. Whey protein (WP = 0.4 g/kg), low Sustamine™ [LSUS = 0.1 g/kg, L-alanylglutamine (AlaGln); Kyowa Hakko Bio. Ltd., New York, NY], high Sustamine™ (HSUS = 0.5 g/kg), and placebo (PLA = 0.52 g/kg glycine, isonitrogenous to the HSUS) were given in randomized order immediately post resistance exercise by intubation. Sixteen rats were used as sedentary controls and received an intubation of glycine (SED: glycine = 0.52 g/kg) at the same time. Rats in each treatment group were subdivided by time of euthanasia, which occurred at 20 or 40 min post intubation (n = 8–13 per group). A second blood sample was collected immediately prior to euthanasia. Following the second blood sample, rats were anesthetized with an intraperitoneal injection of sodium pentobarbital, (75 mg/kg of body weight) during which the flexor hallucis longus (FHL) muscles were excised, freeze clamped in liquid nitrogen, and stored at −80 °C for later analysis. Rats were then euthanized by cardiac injection of sodium pentobarbital (65 mg/kg of body weight).

Blood analysis

From each blood sample, 0.1 ml was withdrawn and immediately transferred to a test tube containing 10 % perchloric acid (PCA). All blood samples and the PCA tubes were centrifuged at 3000g for 10 min at 4 °C with a FS-20 microtube rotor in a Sorvall RC-6 centrifuge (Thermo Fisher Scientific Inc. Waltham, MA). After centrifugation, the cleared plasma samples were transferred to several test tubes and stored at −80 °C for later analysis of glucose, insulin, growth hormone (GH), and IGF-1. The PCA extracts were analyzed for lactate. Plasma glucose was determined using a colorimetric method, which employs glucose oxidase and a modified Trinder color reaction. Plasma insulin was measured using a radioimmunoassay kit (Millipore Corporation, MA) with coefficient of variation (CV) <10 %. Plasma GH was measured using a sandwich ELISA kit with CV < 8 % (Millipore Corporation, MA). The quantification was visualized at dual wavelengths of 450 and 630 nm. Plasma IGF-1 was determined using a high-sensitivity ELISA kit with CV < 10 % (Immunodiagnostic Systems Inc., AZ) and read at dual wavelengths of 450 and 630 nm. Lactate was measured according to Hohorst (1965).

Immunoblot analysis

Immunoblot analysis was performed as previously described (Bernard et al. 2012). In brief, ~80 mg of muscle were homogenized in ice-cold homogenization buffer [20 mM HEPES, 2 mM EGTA, 50 mM (sodium fluoride) NaF, 100 mM potassium chloride (KCl), 0.2 mM EDTA, 50 mM glycerophosphate, 1 mM DL-dithiothreitol (DTT), 0.1 mM phenylmethanesulphonyl fluoride (PMSF), 1 mM benzamidine, and 0.5 mM sodium orthovandate (Na_2VO_4)] at a 1:8 dilution of wet weight muscle with a glass tissue grinder pestle (Corning Life Sciences, Lowell, MA; Caframo Stirrer Type RZR1, Wiarton, Ont. Canada). The homogenates were centrifuged at 14,000g for 10 min at 4 °C, and the supernatants were taken for measurement of total protein concentration and of the phosphorylation of designated cell signaling proteins by immunoblotting. All muscle homogenate aliquots were then stored at −80 °C until analysis.

Muscle samples (60 µg) were combined with an equal amount of Laemmli sample buffer (125 mM Tris, 20 % glycerol, 20 % SDS, 0.25 % bromophenol blue, and β-mercaptoethanol, pH 6.8) and boiled at 95 °C for 10 min in order to denature muscle proteins (Laemmli 1970). Then, samples were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) using 10 % resolving gel at 130 V for 90 min (Bio-Rad Laboratories, Hercules, CA.). The resolved proteins were then electrically transferred onto a nitrocellulose membrane (pore size: 0.45 µm; GE Healthcare Life Sciences, Pittsburgh, PA) using a wet transfer unit (Bio-Rad Laboratories, Hercules, CA) at 90 V for 90 min. Ponceau S. (0.1 % in 0.5 % acetic acid) was used to verify the completeness of the transfer. The membranes were then washed in Tris-buffered saline (TBS) with 0.06 % Tween20 (TTBS) to remove the Ponceau S staining, and then the membranes were blocked in 7 % nonfat milk in TTBS (blocking buffer) for 1 h at room temperature (RT). The membranes were then incubated with the appropriate primary antibody overnight at 4 °C. The targeted phosphorylated proteins were mTOR (Ser2448), p70S6k (Thr389), rpS6 (Ser235/236), protein kinase B (Akt) (Ser473), 5′ adenosine monophosphate-activated protein kinase (AMPK) (Thr172), FOXO3A (Ser318/321), and NF-kB p65 (Ser536). Alpha-tubulin (α-tubulin) was used as an internal loading control. All the antibodies were purchased from Cell Signaling Technology (Cell Signaling Technology, Beverly, MA). Following overnight primary antibody probing, all membranes were washed 5 min, three times with TTBS. Then, the membranes were incubated with HRP-conjugated secondary anti-rabbit IgG (Cell Signaling Technology, Beverly, MA). After three additional 5-min washes, the membranes

were visualized by enhanced chemi-luminescence (ECL) in accordance with the manufacturer's instructions (Perkin Elmer, Boston, MA). All membranes were stripped and re-probed for α-tubulin as an internal loading control. All Western blots were performed in duplicate for each muscle sample to ensure reproducibility (CV < 8 %). Images were captured using a charge-coupled device camera in a Chemi-Doc system (Bio-Rad, Hercules, CA). Intensity of each band was quantified with Quantity One analysis software (Bio-Rad) and expressed as a percentage of a standard.

Statistical analysis

A two-way analysis of variance (ANOVA) was performed to determine significant treatment, time, and treatment by time effects for all blood parameters. When a significant *F* test occurred, differences among means were determined using Fisher's least significance difference (LSD) post hoc analysis. A one-way ANOVA was used for analyzing the data obtained from Western blots, and Fisher's LSD post hoc test was performed to compare mean differences among treatments. Differences with *P* values <0.05 were considered statistically significant. All statistical analyses were performed using IBM SPSS Statistics v19.0 software (IBM Corporation, Armonk), and all data were expressed as mean ± standard error of the mean (SEM).

Results

Blood lactate was significantly elevated immediately post exercise ($p < 0.05$), and then declined to basal level at 20 and 40 min post exercise. Blood lactate did not differ across treatments at 20 or 40 min post exercise ($p > 0.05$) (Table 1). Plasma glucose was not different among groups immediately post exercise ($p > 0.05$). However, plasma glucose was significantly reduced in the WP group both at 20 and 40 min post exercise. At 40 min post exercise, plasma glucose in WP was also significantly lower than that of the SED group ($p < 0.05$) (Table 1). Plasma insulin was transiently increased in SED, PLA, and WP groups at 20 min post exercise ($p < 0.05$), and reduced in the LSUS group (Table 1). However, by 40 min post exercise, insulin levels had returned to immediate post-exercise levels ($p > 0.05$). Plasma GH was significantly reduced by exercise ($p < 0.05$), but was not different than SED by 20 min post exercise. However, 40 min post exercise, GH was

Table 1 Blood lactate, plasma glucose, insulin, GH, and IGF-1 concentration at 0, 20, and 40 min post exercise

Treatment	SED	PLA	WP	LSUS	HSUS
Lactate (mM)					
0 min	1.2 ± 0.1	3.2 ± 0.2*	2.6 ± 0.2*	3.0 ± 0.2*	2.6 ± 0.1*
20 min	1.1 ± 0.2	1.3 ± 0.1†	1.3 ± 0.2†	1.2 ± 0.2†	1.6 ± 0.2†
40 min	1.4 ± 0.1	1.4 ± 0.1†	1.3 ± 0.1†	1.3 ± 0.1†	1.4 ± 0.1†
Glucose (mM)					
0 min	6.2 ± 0.3	6.7 ± 0.2	6.5 ± 0.2	6.4 ± 0.3	6.3 ± 0.2
20 min	5.4 ± 0.4	6.0 ± 0.4	5.1 ± 0.2†	5.7 ± 0.4	5.9 ± 0.3
40 min	6.5 ± 0.2	5.7 ± 0.2 †	5.4 ± 0.3†,*	5.9 ± 0.3	6.1 ± 0.1
Insulin (pM)					
0 min	339.4 ± 38.4	276.8 ± 30.1	292.2 ± 33.5	262.0 ± 24.3	276.5 ± 36.2
20 min	498.2 ± 113.9	394.8 ± 57.2†	418.7 ± 68.8†	220.1 ± 30.1 $^{*,\#,\S}$	356.5 ± 41.5
40 min	364.4 ± 35.5	349.6 ± 44.2	237.0 ± 30.4$^{\#,f}$	215.6 ± 27.8$^{\#}$	281.9 ± 26.9
GH (ng/ml)					
0 min	14.8 ± 4.0	3.3 ± 0.7*	2.6 ± 0.4*	2.5 ± 1.0*	4.5 ± 2.0*
20 min	9.6 ± 4.6	9.1 ± 4.7	12.9 ± 9.0	2.8 ± 0.9	9.7 ± 5.4
40 min	5.1 ± 3.2	9.1 ± 4.1	10.6 ± 6.6	3.0 ± 1.5	19.7 ± 7.0$^{\dagger,*,\#,\P}$
IGF-1 (ng/ml)					
0 min	1071.9 ± 56.5	1017.3 ± 36.6	1073.9 ± 40.0	1062.7 ± 33.7	1045.3 ± 40.8
20 min	958.3 ± 91.4	988.0 ± 55.3	1137.0 ± 77.6	1113.2 ± 92.1	1065.1 ± 97.9
40 min	1093.9 ± 70.9	1071.9 ± 59.5	1127.2 ± 69.7	1094.8 ± 48.1	1112.0 ± 48.7

Data are presented as mean ± SEM ($n = 6$–13 per group)

† $p < 0.05$ vs. 0 min in the same treatment; ^{f} $p < 0.05$ vs. 20 min in the same treatment; * $p < 0.05$ vs. SED at the same time point; $^{\#}$ $p < 0.05$ vs. PLA at the same time point; § $p < 0.05$ vs. WP at the same time point; ¶ $p < 0.05$ vs. LSUS at the same time point

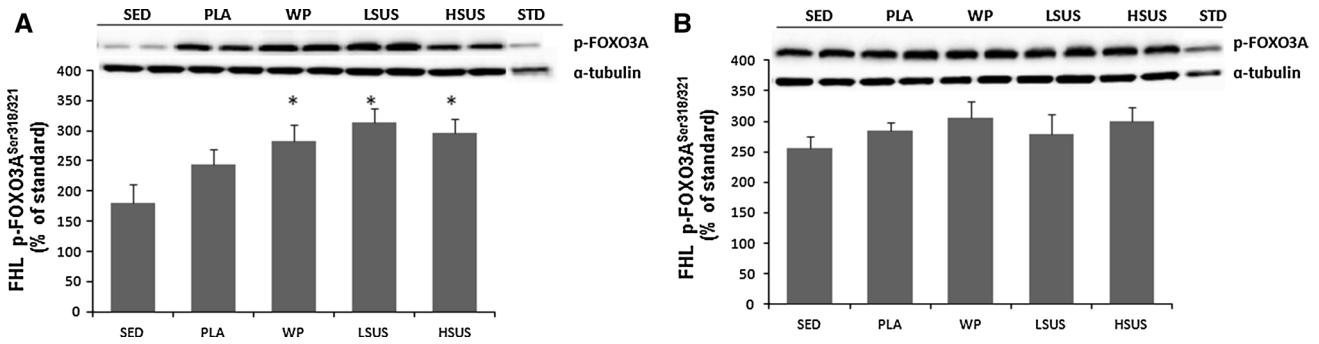

Fig. 1 FOXO3A phosphorylation at $Ser^{318/321}$ expressed as a percentage of an insulin-stimulated rat tissue standard at 20 min (**a**) and 40 min (**b**) post resistance exercise in the FHL muscle. Data are presented as mean ± SEM ($n = 6$–9 per group). *$p < 0.05$ vs. SED

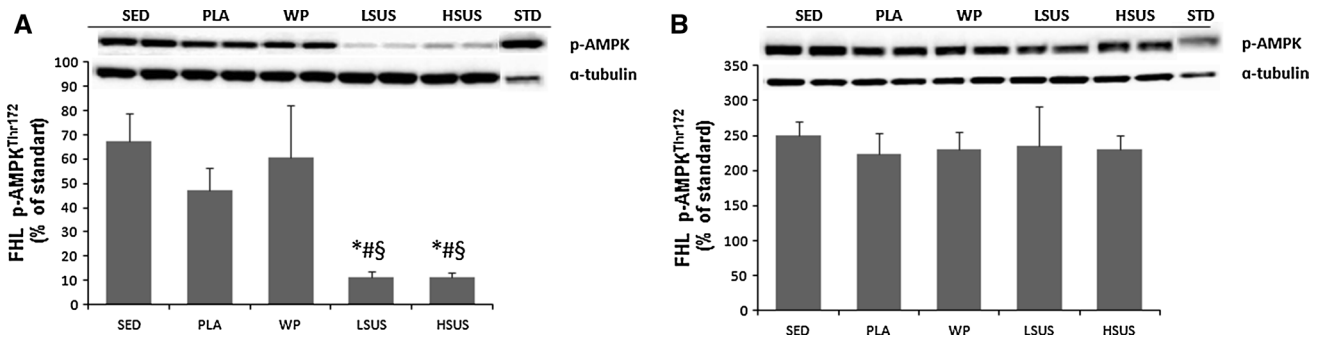

Fig. 2 AMPK phosphorylation at Thr^{172} expressed as a percentage of an insulin-stimulated rat tissue standard at 20 min (**a**) and 40 min (**b**) post resistance exercise in the FHL muscle. Data are presented as mean ± SEM ($n = 6$–9 per group). *$p < 0.05$ vs. SED; #$p < 0.05$ vs. PLA; §$p < 0.05$ vs. WP

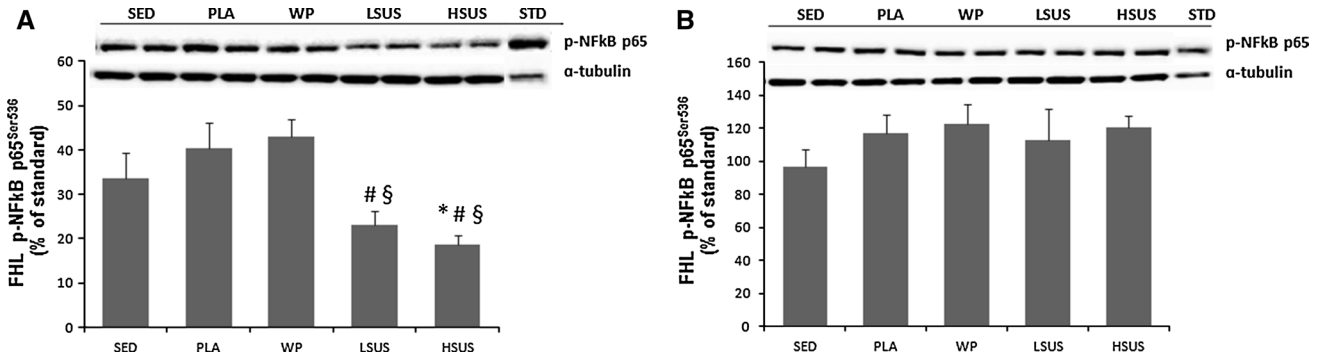

Fig. 3 NF-kB p65 phosphorylation at Ser^{536} expressed as a percentage of an insulin-stimulated rat tissue standard at 20 min (**a**) and 40 min (**b**) post resistance exercise in the FHL muscle. Data are presented as mean ± SEM ($n = 6$–9 per group). *$p < 0.05$ vs. SED; #$p < 0.05$ vs. PLA; §$p < 0.05$ vs. WP

elevated in the HSUS group ($p < 0.05$) above all other treatment groups ($p < 0.05$) (Table 1). There was no difference in plasma IGF-1 across treatments or time (Table 1).

FOXO3A phosphorylation for WP, LSUS, and HSUS was significantly increased above SED at 20 min post exercise ($p < 0.05$) (Fig. 1). At 40 min post exercise, there was no difference across treatment groups ($p > 0.05$) (Fig. 1). The phosphorylation of AMPK and NF-kB p65 was significantly inhibited by LSUS and HSUS at 20 min post exercise compared with SED and PLA ($p < 0.05$). These effects were not seen at 40 min post exercise (Figs. 2, 3). Akt is an upstream substrate of FOXO3A, but the phosphorylation of Akt did not show the same pattern as FOXO3A. Akt phosphorylation was significantly reduced in the WP, LSUS,

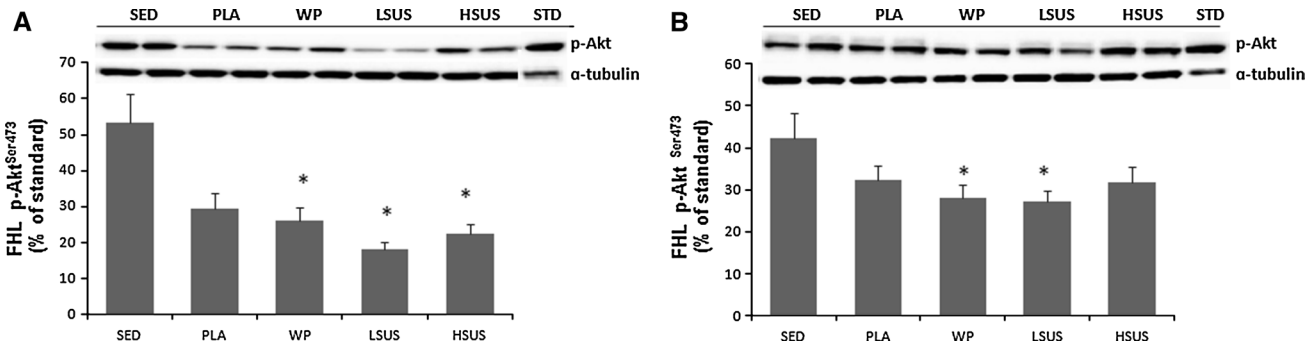

Fig. 4 Akt phosphorylation at Ser^{473} expressed as a percentage of an insulin-stimulated rat tissue standard at 20 min (**a**) and 40 min (**b**) post resistance exercise in the FHL muscle. Data are presented as mean ± SEM ($n = 6$–9 per group). *$p < 0.05$ vs. SED

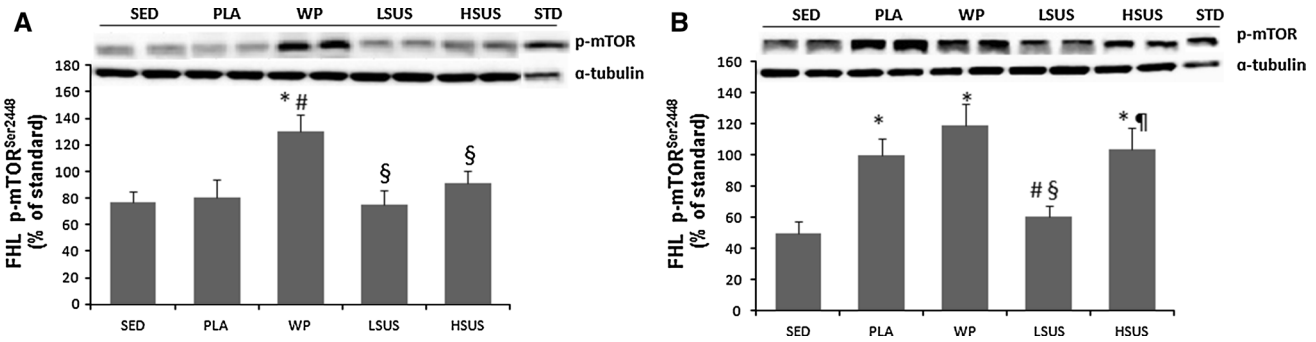

Fig. 5 mTOR phosphorylation at Ser^{2448} expressed as a percentage of an insulin-stimulated rat tissue standard at 20 min (**a**) and 40 min (**b**) post resistance exercise in the FHL muscle. Data are presented as mean ± SEM ($n = 6$–9 per group). *$p < 0.05$ vs. SED; #$p < 0.05$ vs. PLA; §$p < 0.05$ vs. WP; ¶$p < 0.05$ vs. LSUS

and HSUS groups compared with the SED group 20 min post exercise. This reduction in Akt phosphorylation was maintained within the WP and LSUS groups at 40 min post exercise ($p < 0.05$) (Fig. 4).

At 20 min post exercise, the phosphorylation of mTOR was significantly elevated in WP ($p < 0.05$), but not increased in PLA. However, PLA demonstrated a significantly greater mTOR phosphorylation state relative to the SED at 40 min post exercise. Also, at 40 min post exercise, mTOR phosphorylation was significantly increased above SED in WP and HSUS (Fig. 5). Phosphorylation of p70S6k was not increased in PLA at either 20 or 40 min post exercise. However, WP significantly increased the phosphorylation of p70S6k at both 20 and 40 min post exercise above SED and PLA ($p < 0.05$). p70S6k was also activated by LSUS and HSUS above the SED at 40 min post exercise (Fig. 6). Although rpS6 is a downstream target of p70S6k, phosphorylation of rpS6 both at 20 and 40 min post exercise was not enhanced above PLA for LSUS or HSUS. However, WP was able to elicit a greater phosphorylation of rpS6 PLA 20 min post exercise ($p < 0.05$) (Fig. 7).

Discussion

Glutamine, a conditionally essential amino acid, has been shown to play an important role in the regulation of muscle protein turnover under various conditions (Jepson et al. 1988; MacLennan et al. 1987; Zhou and Thompson 1997). When compared with glutamine, AlaGln ingestion produces a higher and more sustained plasma glutamine concentration (Harris et al. 2012). AlaGln has also been found to be more stable in solution than glutamine (Arii et al. 1999; Furst 2001). Positive correlations between plasma glutamine level and protein balance also have been found in studies when a glutamine-containing dipeptide was given to clinical patients (Cruzat et al. 2010; Rogero et al. 2004; Stehle et al. 1989). However, to our knowledge, the present investigation provides the first insight into the phosphorylation states of enzymes controlling MPS and MPB in response to SUS supplementation after resistance exercise.

The primary findings of the present study were that SUS altered, immediately post exercise, the phosphorylation state of signaling proteins in a manner that theoretically should reduce MPB, while WP accelerated the

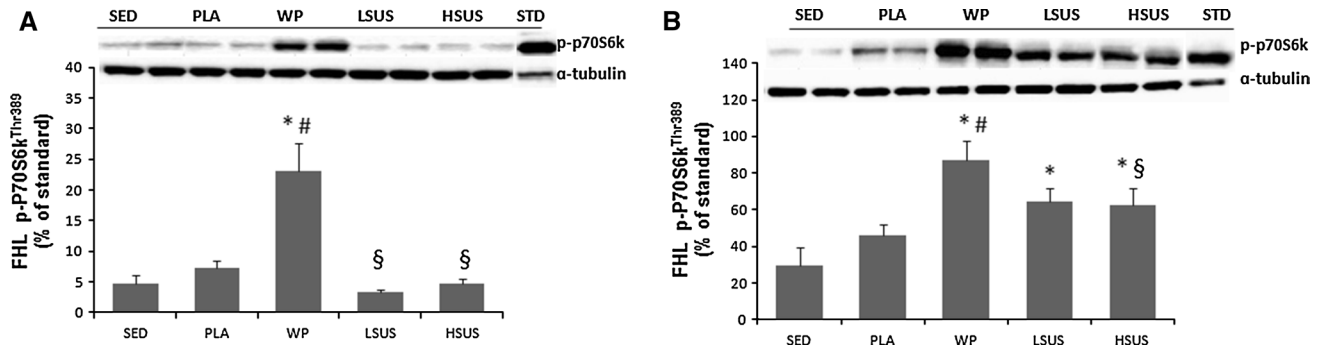

Fig. 6 p70S6k phosphorylation at Thr^{389} expressed as a percentage of an insulin-stimulated rat tissue standard at 20 min (**a**) and 40 min (**b**) post resistance exercise in the FHL muscle. Data are presented as mean ± SEM ($n = 6$–9 per group). *$p < 0.05$ vs. SED; #$p < 0.05$ vs. PLA; §$p < 0.05$ vs. WP

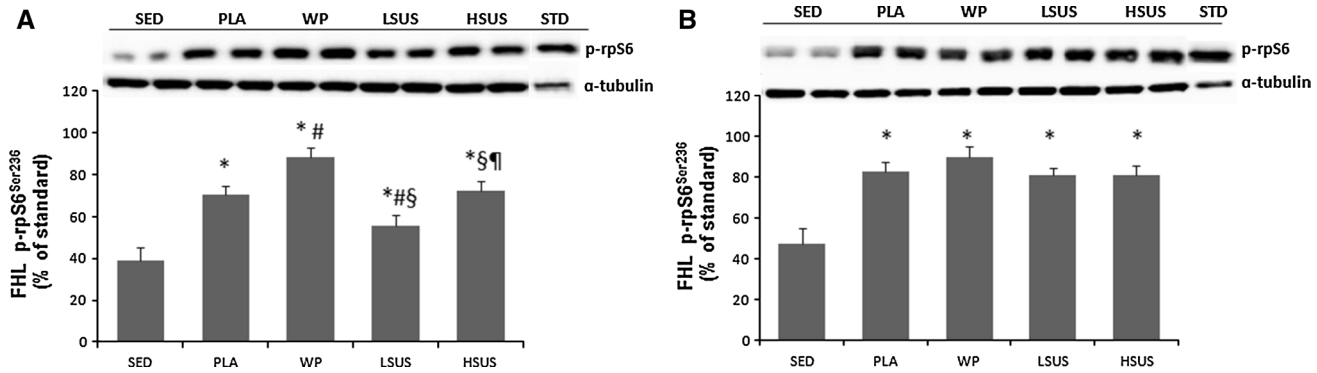

Fig. 7 rpS6 phosphorylation at Ser^{236} expressed as a percentage of an insulin-stimulated rat tissue standard at 20 min (**a**) and 40 min (**b**) post resistance exercise in the FHL muscle. Data are presented as mean ± SEM ($n = 6$–9 per group). *$p < 0.05$ vs. SED; #$p < 0.05$ vs. PLA; §$p < 0.05$ vs. WP; ¶$p < 0.05$ vs. LSUS

phosphorylation of proteins in the mTOR-dependent signaling pathway thereby theoretically activating MPS. With respect to the signaling proteins that control MPB, the principal proteolytic systems in skeletal muscle are classified into the ubiquitin–proteasome system, lysosomal proteolysis, and Ca^{2+}-activated proteases (i.e. calpain) (Jagoe and Goldberg 2001; Powers et al. 2007). Among these proteolytic systems, the ubiquitin–proteasome system is the primary signaling pathway that mediates myofibrillar protein degradation (Jagoe and Goldberg 2001). Two crucial ubiquitin E3 ligases in the skeletal muscle, muscle atrophy F-box (MAFbx or atrogin-1) and muscle ring-finger protein 1 (MuRF-1), have been shown to stimulate muscle proteolysis (Attaix et al. 2005). The activation of these ubiquitin E3 ligases is under the regulation of FOXO3A, a critical transcription factor in the nucleus (Zanchi et al. 2010). In the present study, WP and two doses of SUS yielded a transient increase in the phosphorylation of FOXO3A above the sedentary level at 20 min post exercise. Phosphorylated FOXO3A can be exported into the cytoplasm where it becomes inactive, and thereby reducing the expression of the ubiquitin E3 ligases in the nucleus. Akt, which is activated by insulin/IGF-1 via the phosphatidylinositol 3-kinase (PI3k) pathway, is considered as an important regulator of FOXO3A phosphorylation (Brunet et al. 1999). Nevertheless, we found that the phosphorylation of Akt was significantly reduced at 20 min post exercise when WP, LSUS, and HSUS supplements were provided and at 40 min post exercise. Therefore, it seems unlikely that the insulin–Akt pathway was involved in the phosphorylation of FOXO3A in the present study. Rather, we found that the phosphorylation of AMPK was significantly reduced by both doses of SUS at 20 min post exercise, which was inversely related to the phosphorylation of FOXO3A. AMPK, an important energy sensor, is another upstream regulator of FOXO3A. Research has demonstrated that activation of AMPK can increase myofibrillar protein degradation by increasing the expression of atrogin-1 and MuRF-1 in C2C12 myotubes secondary to increasing the expression of FOXO3A (Nakashima and Yakabe 2007). Glutamine starvation in cells results in an increase in the phosphorylated to total AMPK cellular content (Zhu et al. 2014).

Moreover, glutamine supplementation has been found to attenuate MPB and muscle atrophy by blunting atrogin-1 and MuRF-1 expression (Bonetto et al. 2011; Lambertucci et al. 2012). Therefore, it is reasonable to assume that the phosphorylation of FOXO3A following SUS supplementation was mediated by the inhibition of AMPK, which in turn would likely reduce expression of atrogin-1 and MuRF-1 and prevent MPB after resistance exercise.

It is noteworthy that the phosphorylation of NF-kB p65 was also transiently reduced by both doses of SUS at 20 min post exercise relative to the placebo and WP groups. Cai et al. (2004) reported that the activation of the NF-kB p65 signaling pathway leading to muscle loss was due to the activation of MuRF-1. Accordingly, the inhibition of NF-kB p65 phosphorylation by SUS might be another way of exerting an inhibitory effect on MPB post exercise. Interestingly, the phosphorylation states of AMPK and NF-kB p65 were not influenced by the WP supplement. Previous research suggests that protein ingestion can promote MPS without changing MPB relative to exercise alone (Borsheim et al. 2002; Miller et al. 2003; Tang et al. 2009), and our results support this position. Taken together, we suggest that SUS supplementation post exercise will inhibit MPB via suppressing the activation of AMPK-FOXO3A and NF-kB p65, whereas WP has a negligible effect on the signaling pathways that control MPB.

With respect to the signaling proteins that control MPS, the activation of mTOR stimulates the phosphorylation of p70S6k (Fingar et al. 2002), which then phosphorylates its downstream substrate protein rpS6 resulting in translation of mRNA to increase the capacity for protein synthesis (Jastrzebski et al. 2007). In the current investigation, WP provided immediately after resistance exercise yielded a greater increase in the phosphorylation states of mTOR, p70S6k, and rpS6 relative to the placebo and sedentary groups at 20 min post exercise. These results are in concert with a previous study from our laboratory demonstrating that provision of whey isolate after endurance exercise transiently enhanced the phosphorylation of mTOR and p70S6k in the quadriceps muscles of rats (Morrison et al. 2008). Similar results have also been found in human subjects when they were supplemented with whey protein post resistance exercise (Hulmi et al. 2009).

When no supplement was provided, an increased phosphorylation of mTOR was delayed until 40 min post exercise. Interestingly, exercise alone resulted in the phosphorylation of rpS6 at both 20 and 40 min post exercise. However, the phosphorylation of p70S6k was not affected. In agreement with these results, Bolster et al. (2003) found an increase in rpS6 phosphorylation in response to resistance exercise without changing the phosphorylation status of p70S6k. Aside from being phosphorylated by p70S6k, rpS6 can be directly phosphorylated by 90-kDa ribosomal S6 kinase ($p90^{RSK}$), a component of the extracellular signal-regulated kinase (ERK) 1/2 cascade (Pende et al. 2004; Roux and Blenis 2004). Therefore, activation of ERK 1/2 by muscle contraction may explain how exercise alone activated rpS6 post exercise without affecting the phosphorylation of p70S6k.

Although MPS was not directly measured in the present study, protein/AAs supplementation has been clearly shown to stimulate MPS both at rest (Atherton et al. 2010) and after different types of exercise (Anthony et al. 2007; Borsheim et al. 2002; Moore et al. 2009; Tipton et al. 1999). This increase in protein synthesis has been correlated with the activation of the mTOR signaling pathway and modulation of its downstream target proteins involved in mRNA translation initiation (Bolster et al. 2004; Goodman et al. 2010). Therefore, our results indicate that WP supplementation soon after exercise is capable of hastening the activation of the mTOR signaling pathway and muscle protein synthesis. Akt is an upstream activator of mTOR (Saltiel 1996) and is activated by insulin via the PI-3 kinase pathway. However, we found that although WP transiently increased the plasma insulin level, the phosphorylation of Akt was significantly decreased below the SED level. Accordingly, the activation of the mTOR signaling pathway by the WP supplement does not appear to have been as a result of the activation insulin-PI3k-Akt pathway. Moreover, neither exercise nor WP influenced the plasma IGF-1 level suggesting this hormone also was not related to the phosphorylation of mTOR and its downstream target proteins. It is likely that the influence exerted over the mTOR pathway by the WP supplement was due to leucine activation of mTOR. Leucine is a strong independent activator of mTOR and a prominent amino acid in whey (Anthony et al. 2001, 2007; Bolster et al. 2003).

It was of interest to note that both doses of SUS increased p70S6k phosphorylation relative to the SED at 40 min post exercise while p70S6k phosphorylation for placebo was not. The activation of p70S6k has been suggested to correlate with muscle hypertrophy (Baar and Esser 1999), and therefore suggests that SUS may potentially impact MPS through activating p70S6k. Plasma GH level dampened immediately after exercise was elevated above the basal level by HSUS at 40 min post exercise. Early investigations demonstrated that plasma concentration of GH can be elevated by the administration of glutamine (Welbourne 1995), and that plasma GH was associated with skeletal MPS (Fryburg and Barrett 1993; Fryburg et al. 1991). However, a recent study reported no effect of systemic concentration of anabolic hormones on MPS and phosphorylation of signaling proteins (West et al. 2009), but suggested local anabolic hormones may predominate MPS post exercise. Moreover, the present study showed that phosphorylation states of mTOR and rpS6 were not

influenced by SUS at either 20 or 40 min. Although SUS increased the phosphorylation of p70S6k, it appears that SUS only has a slight impact on the mTOR signaling pathway. A recent study demonstrated that the modulation of glutamine supplementation on expressions of mRNA and proteins controlling MPS and MPB was seen in diabetic rats but not in normal non-diabetic rats (Lambertucci et al. 2012). In an in vitro study conducted by Zhou and colleagues (1997), it was observed that the rate of protein synthesis after glutamine administration was only seen in stressed myotube (administered by a heat shock treatment) but not in normal cultured myotube indicating that the effect of glutamine on protein synthesis may be conditionally dependent. Perhaps our SUS supplementation would be more effective in stimulating MPS under states of stress when catabolism is high such as following muscle injury, severe disease or burns, or during over training when the glutamine level falls below its normal physiological level.

In conclusion, the results of the current study provide indirect evidence that SUS inhibits MPB via suppressing activation of AMPK-FOXO3A and NF-kB p65 post exercise, whereas WP appears to accelerate the activation of the mTOR signaling pathway and promote MPS. The actions of WP on the phosphorylation of mTOR did not appear to be hormonally controlled, but were most likely due to the actions of leucine, a prominent amino acid in whey. Together, these findings suggest that a combination of WP and SUS supplementation post exercise might result in the phosphorylation of metabolic regulatory enzymes in a manner that would increase MPS and decrease MPB, thereby maximizing muscle protein accretion.

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