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Effects of resistance training and protein plus amino acid supplementation on muscle anabolism, mass, and strength

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Summary. This study examined 10 wks of resistance training and the ingestion of supplemental protein and amino acids on muscle performance and markers of muscle anabolism. Nineteen untrained males were randomly assigned to supplement groups containing either 20 g protein (14 g whey and casein protein, 6 g free amino acids) or 20 g dextrose placebo ingested 1 h before and after exercise for a total of 40 g/d. Participants exercised 4 times/wk using 3 sets of 6–8 repetitions at 85–90% of the one repetition maximum. Data were analyzed with two-way ANOVA ($p < 0.05$). The protein supplement resulted in greater increases in total body mass, fat-free mass, thigh mass, muscle strength, serum IGF-1, IGF-1 mRNA, MHC I and IIa expression, and myofibrillar protein. Ten-wks of resistance training with 20 g protein and amino acids ingested 1 h before and after exercise is more effective than carbohydrate placebo in up-regulating markers of muscle protein synthesis and anabolism along with subsequent improvements in muscle performance.

Keywords: Resistance training – Insulin – Myosin heavy chain – IGF-1 – Amino acids

Introduction

In humans, provided the exercise intensity is of sufficient magnitude, acute changes in the rate of skeletal muscle protein turnover can occur resulting in increases in both protein synthesis and degradation (Phillips et al., 1997). A single bout of resistance exercise is also capable of up-regulating muscle-specific gene expression (Willoughby and Nelson, 2002), thereby supporting the contention that muscle hypertrophy occurring with frequent bouts of resistance exercise over the course of the training period can be partially explained by pre- and post-translational mechanisms. However, in the absence of nutritional intake muscle protein degradation may exceed synthesis in the early stages of recovery from exercise (Biolo et al., 1997). Carbohydrate supplementation during recovery from resistance exercise has been suggested to reduce muscle protein degradation with no effect on muscle protein synthesis (Roy et al., 1997, 2000). Furthermore, carbohydrate supplementation in the recovery phase of resistance exercise improved net protein balance but the effect was less than that of ingested amino acids (Borsheim et al., 2004b). However, it has been shown that exercise-induced muscle protein synthesis can be exacerbated by protein and/or amino acid intake immediately before and after resistance exercise (Borsheim et al., 2004a; Carroll et al., 2005; Rasmussen et al., 2000). In addition, the availability of protein and/or amino acids during recovery from resistance exercise significantly enhances muscle protein synthesis while having the opposite effect on muscle protein degradation (Biolo et al., 1997).

Protein supplementation and the subsequent availability of circulating amino acids in conjunction with a resistance-training program is also associated with elevations in serum insulin and IGF-1, both of which are known to augment muscle protein synthesis (Ballard et al., 2005). The sarcomeric proteins primarily synthesized during and after resistance exercise appear to be those that comprise the contractile machinery (Jones and Rutherford, 1987), as evidenced by changes in the expression of myosin heavy chain (MHC) protein isoform mRNA and protein (Willoughby and Nelson, 2002).

In terms of protein supplementation supporting, and potentially enhancing the protein synthetic process involved in muscle hypertrophy, the quality of protein and

the timing of ingestion may be of significance. Despite different patterns of blood amino acid responses, the acute ingestion of both whey and casein protein 1 h after resistance exercise resulted in similar increases in muscle protein net balance and net muscle protein synthesis (Tipton et al., 2004). Whey protein is considered a "fast blend" and is based on the premise that when ingested it will instigate a rapid increase in blood levels of amino acids. On the other hand, casein protein is considered a "slow blend" and will manifest a slow, sustained increase in blood amino acids for several hours (Dangin et al., 2001). As a result, the combination of whey and casein protein may be beneficial in supporting muscle protein synthesis and increasing muscle mass during the course of a heavy resistance-training program. For example, 14-wks of resistance training supplemented with a blend of whey and casein has been shown to result in Types I and II muscle fiber hypertrophy and improved muscle performance (Andersen et al., 2005).

It is not conclusive as to whether carbohydrate supplementation can directly increase muscle protein synthesis. However, it seems clear that protein and amino acid administration can increase muscle protein synthesis and appears that an intake of protein and amino acids immediately before and after resistance exercise may exacerbate this process. Therefore, the purpose of this study was to compare the effects of a 10-wks heavy resistance-training program combined with the ingestion of a whey and casein protein and free amino acids (essential and non-essential) supplement versus a carbohydrate placebo supplement on muscle strength and mass and systemic and local factors indicative of muscle anabolism in untrained males.

Methods

Participants

Twenty untrained males with an average (±SD) age, height, and total body mass of 19 (±1.58) yrs, 68.99 (±4.02) in, and 81.72 (±16.15) kg, respectively, participated in the 10-wk study. However, one subject was forced to withdraw from the study due to illness unrelated to the study. Subjects with contraindications to exercise as outlined by the American College of Sports Medicine (ACSM, 2000) and/or who had consumed any nutritional supplements 3 months prior to the study were excluded from participation. All eligible subjects signed university-approved informed consent documents and approval was granted by the Institutional Review Board for Human Subjects. Additionally, all experimental procedures involved in the study conformed to the ethical consideration of the Helsinki Code. After baseline testing, participants were matched by age, total body mass, and leg press strength, and then randomly assigned, in a double blind fashion, to a resistance exercise group combined with either a carbohydrate placebo supplement [PLC ($n=9$)] or a protein and amino acid supplement [PRO ($n=10$)].

Muscle biopsies and venous blood sampling

Percutaneous muscle biopsies (50–70 mg) were obtained prior to supplement ingestion before the exercise session at wks 1 and 24 h following the last exercise session at the end of wk 10. Muscle samples were taken from the middle portion of the right vastus lateralis muscle at the midpoint between the patella and the greater trochanter of the femur at a depth between 2 and 3 cm. For the final biopsy, attempts were made to extract tissue from approximately the same location as the initial biopsy by using the pre-biopsy scar, depth markings on the needle, and a successive incision that was made approximately 0.5 cm to the former from medial to lateral. Muscle specimens were immediately frozen in liquid nitrogen and then stored at −80 °C for later analysis.

Venous blood samples were obtained from the antecubital vein into 10 ml collection tubes. Blood samples were allowed to stand at room temperature for 10 min and then centrifuged. The serum was then removed and frozen at −20 °C for later analysis. For IGF-1, blood samples were obtained prior to supplement ingestion before the exercise session at wks 1 and 24 h following the last exercise session at the end of wk 10. For insulin, blood samples were obtained immediately prior to ingesting the supplement and immediately prior to the exercise session at wk 1 and 10. All blood samples were obtained after an 8 h fast standardized to the same time of day for each sample.

Strength testing

Five days before training at wk 1 and immediately prior to the final exercise session at the end of wk 4 and 8, and 24 h after the final exercise session at wk 10 both groups were subjected to a testing session in which each participant's one repetition maximum (1-RM) was assessed on each of the exercises used in the training program. However, bench press and leg press strength were the exercises used to evaluate upper- and lower-body strength, respectively, and relative strength (strength/body weight) was used as the criterion strength variable because it corrects for variations in body mass among subjects, thereby providing a more accurate estimate of strength (Baechle et al., 2000; Willoughby, 2004a, b; Willoughby and Rosene, 2001).

Anthropometrics and body composition testing

Total body mass, total body water, percent body fat, fat mass, fat-free mass, and thigh mass were determined during the initial strength testing session five days prior to the first exercise session at wks 1 and 24 h following the final exercise session at the end of wk 10. Total body mass (kg) was determined on a standard dual beam balance scale (Detecto, Terre Haute, IN, USA). Thigh mass (kg) was calculated taking into account surface measurements of the length, circumference, and skin fold thickness of each subject's dominant thigh (Andersen and Saltin, 1985). The measurements were performed in the supine position and always prior to exercise to avoid the influence of possible exercise-induced muscle swelling. Percent body fat was determined using hydrostatic weighing. Test-retest reliability of performing these assessments on participants in our laboratory has yielded low mean coefficients of variation and high reliability for the determination of thigh mass (1.7%, intraclass, $r=0.95$) and percent body fat (1.9% intraclass, $r=0.93$). Total body water was determined using a Tanita BF-350E bioelectrical impedance analyzer (Tanita Corporation, Arlington Heights, IL, USA).

Training protocol

Both groups engaged in four resistance-training sessions each wk with the upper-body being trained on Monday and Thursday (bench press, shoulder press, lat pulldown, tricep pressdown, bicep curl, and abdominal crunch exercises) and lower-body on Tuesday and Friday (leg press, knee extension, knee curl, and calf raise exercises) incorporating the principles of overload and progressive resistance (Willoughby, 2004a, b; Willoughby and Rosene, 2001). Each exercise session lasted approximately 45 min/session

(excluding 10 min of warm-up and cool-down), and all sessions were supervised by the principal investigator and/or trained graduate student personnel. The format and relative intensity for the training protocol involved 3 sets of 6–8 repetitions at 85–90% 1-RM. Participants were allowed 180 sec of rest between sets and exercises, as this represented the appropriate work:rest ratio (1:5) for this exercise intensity (Baechle et al., 2000). Missed training sessions were made up on an alternate day and in such a way that a standard 48 h existed between sessions to allow for adequate recovery and recuperation between sessions. Subjects were informed that missing three training sessions would result in disqualification from the study. In addition to the pre-training 1-RM for PLC and PRO, the 1-RM was assessed after wks 4 and 8 for all training exercises so that any necessary adjustments could be made to accommodate for strength increases, thereby ensuring that subjects continued to train at a relative intensity of 85–90% of their 1-RM.

Supplementation protocol

At each exercise session participants were provided their respective supplement mixed with 500 ml of water 1 h before and immediately after exercise. The PLC group received a total of 40 g of dextrose, whereas the PRO group received a total of 40 g protein [28 g of protein (14 g whey protein concentrate, 6 g whey protein isolate, 4 g milk protein isolate, 4 g calcium caseinate) and 12 g of free amino acids (0.22 g arginine, 0.22 g histidine, 0.14 g isoleucine, 6.0 g leucine, 0.44 g lysine, 0.44 g methionine, 0.20 g phenylalanine, and 0.22 g valine, 0.12 g aspartate, 2.0 g glutamine, and 2.0 g tyrosine)]. Both supplements were isocaloric and independently prepared in individually blinded packages (FSI Nutrition, Omaha, NE, USA). On non-exercise days, 40 g of the PLC or PRO was ingested in the morning upon waking. Participants were supplied with the appropriate number of supplement packages for non-exercise days on a weekly basis and returned the empty packages at the end of each week to help insure compliance.

Dietary records

The subjects' diets were not standardized and subjects were asked not to change their dietary habits during the course of the study. However, subjects were required to keep 7-d dietary records during the week prior to beginning the training and supplementation protocol and also during wks 5 and 10. The dietary recalls were evaluated with the Food Processor dietary assessment software program (ESHA Research, Salem, OR, USA) to determine the average daily macronutrient consumption of fat, carbohydrate, and protein in the diet.

Skeletal muscle total RNA isolation

Total cellular RNA was extracted from biopsy samples with a monophasic solution of phenol and guanidine isothiocyanate (Chomczynski and Sacchi, 1987) contained within the TRI-reagent (Sigma Chemical Co., St. Louis, MO). Aliquots of total RNA were then separated with agarose gel electrophoresis and monitored under an ultraviolet light (Chemi-Doc XRS, Bio-Rad, Hercules, CA) to verify RNA integrity and absence of RNA degradation, indicated by prominent 28s and 18s ribosomal RNA bands (data not shown), as well as an OD_{260}/OD_{280} ratio of approximately 2.0 (Current Protocols in Molecular Biology, 1999; Willoughby, 2004a, b; Willoughby and Nelson, 2002; Willoughby and Rosene, 2001). We observed an average ($\pm$SD) ratio of 1.93 ($\pm$0.03) for all samples. The RNA samples were stored at $-80\,^{\circ}$C until later analysis.

Reverse transcription and cDNA synthesis

Two micrograms of total skeletal muscle RNA were reverse-transcribed to synthesize cDNA using the iSscript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Each reverse transcription reaction mixture was incubated at 25 °C for 5 min, 42 °C for 30 min, heated to 85 °C for 10 min, and then quick-chilled on ice. The cDNA concentration was determined by using an OD_{260} equivalent to 50 μg/μl (Current Protocols in Molecular Biology, 1999), and starting cDNA template concentration was standardized by adjusting all samples to 200 ng prior to amplification (Willoughby, 2004a, b).

Oligonucleotide primers for PCR

The mRNA sequences of human skeletal muscle β-actin (NM_001101), Type I MHC (AC X06976), Type IIa MHC (AF111784), Type IIx MHC (AF111785), IGF-1Ea (NM_000618), and β-actin (NM_001101) published in the NCBI Entrez Nucleotide database (www.ncdi.nlm.hih.gov) were used to construct PCR primers using Beacon Designer software (Bio-Rad, Hercules, CA, USA), and then commercially synthesized (Integrated DNA Technologies, Coralville, IA). These primers amplify fragments of 141, 145, and 148 bp, respectively, for Types I, IIa, IIx MHC, and 150 bp for IGF-1. Due to its consideration as a constitutively expressed "housekeeping gene," and the fact that it has been shown to be an appropriate external reference standard in real-time PCR, β-actin was used for detecting the relative change in the quantity of mRNA (Mahoney et al., 2004). For β-actin, these primers amplify a PCR fragment of 135 bp. Additionally, we observed β-actin to undergo only a small amount of variation in expression from one sampling point to the next. The overall average variation between the two muscle samples was 2.25%, and provides further evidence to suggest β-actin as an appropriate external control.

Real-time PCR amplification and quantitation

Two hundred nanograms of cDNA template were added to iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) and each PCR reaction was amplified using real-time quantitative PCR (iCycler IQ Real-Time PCR Detection System, Bio-Rad, Hercules, CA, USA). The amplification profile was run for 40 cycles employing a denaturation step at 95 °C for 30 sec, primer annealing at 58 °C for 30 sec, and extension at 72 °C for 30 sec. Fluorescence was measured after each cycle resulting from the incorporation of SYBR green dye into each amplicon. The quantity of mRNA was determined relative to the expression of β-actin, and ΔC_T values were used to compare gene expression before and after the resistance training and supplementation protocols. The specificity of the PCR was demonstrated with an absolute negative control reaction containing no cDNA template, and a single gene product was confirmed using DNA melt curve analysis. Positive amplification of the amplicons was assessed with agarose gel electrophoresis illuminated with UV transillumination (Chemi-Doc XRS, Bio-Rad, Hercules, CA, USA) (data not shown).

Myofibrillar protein quantitation

Total protein remaining after the total RNA isolation procedure was isolated with isopropanol, ethanol, and 0.3 M guanidine hydrochloride. Myofibrillar protein was further isolated with repeated incubations in 0.1% SDS at 50 °C and separated by centrifugation. Myofibrillar protein content was determined spectrophotometrically based on the Bradford method at a wavelength of 595 nm (Bradford, 1976). A standard curve was generated ($r^2 = 0.98$, $p = 0.001$) using bovine serum albumin (Bio-Rad, Hercules, CA) and myofibrillar protein was quantified relative to muscle wet-weight (Willoughby and Nelson, 2002; Willoughby, 2004b).

MHC protein isoform quantitation

Using the principles of SDS-PAGE and LabChip (Caliper Life Sciences, Hopkinton, MA) technology, the MHC protein isoform composition within 20 μg muscle homogenates was determined under denaturing conditions by using an Experion Pro260 automated electrophoresis system (Bio-Rad, Hercules, CA). The Experion Pro260 analysis kit has a resolution and quantitation of 10–260 kDa proteins while also separating and detecting 2.5–2000 ng/μl protein. The Experion Pro260 system combines

electrophoresis, staining, destaining, imaging, band detection, and basic data analysis into a single, automated step. Gel images were then processed and displayed on a computer monitor and MHC bands identified by migration relative to the molecular weight marker (data not shown). The density of the MHC bands was determined using Experion Imaging software (Bio-Rad, Hercules, CA), expressed in arbitrary density units.

Serum insulin and IGF-1 quantification

The concentrations of serum insulin and IGF-1 were determined in duplicate and the average concentrations reported using commercially available ELISA kits (Diagnostics Systems Laboratories, Webster, TX, USA). A standard curve was generated for insulin ($r^2 = 0.99$, $p = 0.001$) and IGF-1 ($r^2 = 0.99$, $p = 0.001$) using specific, serially diluted control antigens contained in the kit. The concentrations of insulin and IGF-1 were determined at an optical density of 450 nm with a microplate reader (Wallac Victor 1420, Perkin Elmer, Boston, MA).

Statistical analysis

Data was analyzed by utilizing separate 2 × 3 [Group (CHO, PRO)] × Test (wk 0, wk 5, wk 10) mixed design factorial analyses of variance (ANOVA) for the dietary intakes, whereas a 2 × 2 [Group (CHO, PRO)] × Test (wk 0, wk 10) factorial mixed design ANOVA with repeated measures for all other criterion variables. If a significant group, test, and/or interaction was observed, least significant differences (LSD) analyses were performed to determine where significance was obtained. However, to protect against Type I error, the conservative Hunyh-Feldt Epsilon correction factor was used to evaluate observed within-group F-ratios. All data was considered statistically significant when the probability of Type I error was ≤0.05 and power analysis of the design indicates that an n-size of 10 per group yields high power (>0.80) for delta values of 0.80–1.25.

Results

Macronutrient intake

There were no significant main effects for Group ($p = 0.567$) or Test ($p = 0.423$) or the Group × Test interaction ($p = 0.783$) located for the average daily intake of carbo-

Table 1. Average (±SD) daily macronutrient intakes of the CHO and PRO groups prior to and after 5 and 10 weeks of resistance training and supplementation

Group	Carbohydrate[a]	Protein[a]	Fat[a]
PLC			
Pre	4.18 (0.56)	2.06 (0.68)	1.32 (0.41)
5-wks	4.37 (0.62)	2.18 (0.78)	1.42 (0.71)
10-wks	4.24 (0.76)	2.24 (0.85)	1.39 (0.72)
PRO			
Pre	4.38 (0.79)	2.15 (0.86)	1.41 (0.50)
5-wks	4.52 (0.74)	2.26 (0.62)	1.75 (0.65)
10-wks	4.64 (0.89)	2.31 (0.82)	1.69 (0.59)

[a] Average daily macronutrient intakes are based on 7-day food records collected the wk prior to training and supplementation and during wks 5 and 10. Intake values do not include the ingestion of either of the two supplements, and are expressed in g/kg total body mass. No significant differences in macronutrient intake were observed between the PLC and PRO groups throughout the course of the study ($P > 0.05$)

Table 2. Mean (±SD) body composition and delta values for the PLC and PRO groups prior to (Wk 0) and after 10 wks of resistance training and supplementation

Variable	PLC	PRO	Group	Test	Group × Test
Body Mass (kg)					
Wk 0	78.63 (13.64)	81.46 (15.78)			
Wk 10	82.98 (16.52)[a]	88.43 (18.10)[a,b]			
ΔBody Mass	4.35 (2.88)	7.00 (2.32)	$p = 0.030$	$p = 0.041$	$p = 0.297$
Body Water (kg)					
Wk 0	46.76 (15.78)	46.05 (6.85)			
Wk 10	49.22 (18.10)	49.44 (7.50)			
ΔBody Water	2.46 (1.32)	3.39 (0.65)	$p = 0.124$	$p = 0.853$	$p = 0.545$
Body Fat (%)					
Wk 0	19.95 (6.94)	21.52 (7.14)			
Wk 10	18.88 (8.10)	19.46 (6.75)			
Δ Body Fat	−1.07 (1.16)	−2.06 (0.39)	$p = 0.494$	$p = 0.417$	$p = 0.998$
Fat Mass (kg)					
Wk 0	16.15 (7.64)	18.81 (10.11)			
Wk 10	15.93 (7.40)	17.68 (8.29)			
Δ Fat Mass	−0.22 (0.24)	−1.13 (0.82)	$p = 0.821$	$p = 0.431$	$p = 0.758$
Fat-Free Mass (kg)					
Wk 0	61.15 (10.57)	63.22 (9.35)			
Wk 10	63.85 (11.88)[a]	68.84 (10.33)[a,b]			
Δ Fat-Free Mass	2.70 (1.31)	5.62 (0.98)	$p = 0.046$	$p = 0.034$	$p = 0.535$

Group = main effect for Group factor; Test = main effect for Test factor; Group × Test = interaction
[a] $p \leq 0.05$ from wk 0
[b] $p \leq 0.05$ from PLC

Table 3. Mean (±SD) muscle strength and mass, myofibrillar protein, and delta values for the PLC and PRO groups prior to (wk 0) and after 10 wks of resistance training and supplementation

Variable	PLC	PRO	Group	Test	Group × Test
BP Strength (kg/kg)					
Wk 0	0.89 (0.23)	0.91 (0.18)			
Wk 10	1.09 (0.15)[a]	1.39 (0.20)[a,b]			
Δ BP Strength	0.20 (0.08)	0.48 (0.02)	$p=0.035$	$p=0.009$	$p=0.778$
LP Strength (kg/kg)					
Wk 0	2.78 (0.86)	2.96 (0.41)			
Wk 10	3.39 (0.89)[a]	4.09 (0.53)[a,b]			
Δ LP Strength	0.61 (0.03)	1.13 (0.12)	$p=0.015$	$p=0.010$	$p=0.674$
Thigh Mass (kg)					
Wk 0	2.72 (0.66)	2.92 (0.65)			
Wk 10	3.13 (0.69)[a]	3.65 (0.73)[a,b]			
Δ Thigh Mass	0.41 (0.03)	0.73 (0.08)	$p=0.022$	$p=0.025$	$p=0.437$
Myo Protein (μg/mg)					
Wk 0	60.02 (10.83)	55.31 (15.18)			
Wk 10	70.56 (12.47)[a]	80.34 (18.11)[a,b]			
Δ Protein	10.54 (2.35)	25.03 (5.78)	$p=0.029$	$p=0.006$	$p=0.633$

BP Bench press; *LP* leg press; *Myo protein* myofibrillar protein; Group = main effect for Group factor; Test = main effect for Test factor; Group × Test = interaction
[a] $p \leq 0.05$ from wk 0
[b] $p \leq 0.05$ from PLC

hydrate, fat, and protein during the course of the 10 wk resistance training and supplementation period (Table 1).

Body composition and anthropometric variables

There were no significant Group × Test interactions for total body mass ($p=0.297$), fat-free mass ($p=0.535$), and thigh mass ($p=0.437$). However, significant main effects for Group were located for total body mass ($p=0.030$), fat-free mass ($p=0.046$), and thigh mass ($p=0.022$). In addition, significant main effect for Test were observed for total body mass ($p=0.041$), fat-free mass ($p=0.034$), and thigh mass ($p=0.025$). Results showed respective increases in total body mass, fat-free mass, and thigh mass of 4.35 ± 2.88, 2.70 ± 1.31, and 0.41 ± 0.03 kg for PLC and increases of 7.00 ± 2.32, 5.62 ± 0.98, and 0.73 ± 0.08 kg for PRO. These data indicate that both groups underwent significant increases in these variables over the 10-wk period; however, the increases for PRO were greater than PLC (Tables 2 and 3).

Relative muscle strength variables

There were no significant Group × Test interactions for bench press strength ($p=0.778$) and leg press strength ($p=0.674$). However, significant main effects for Group were observed for bench press ($p=0.035$) and leg press strength ($p=0.015$). Significant main effects for Test

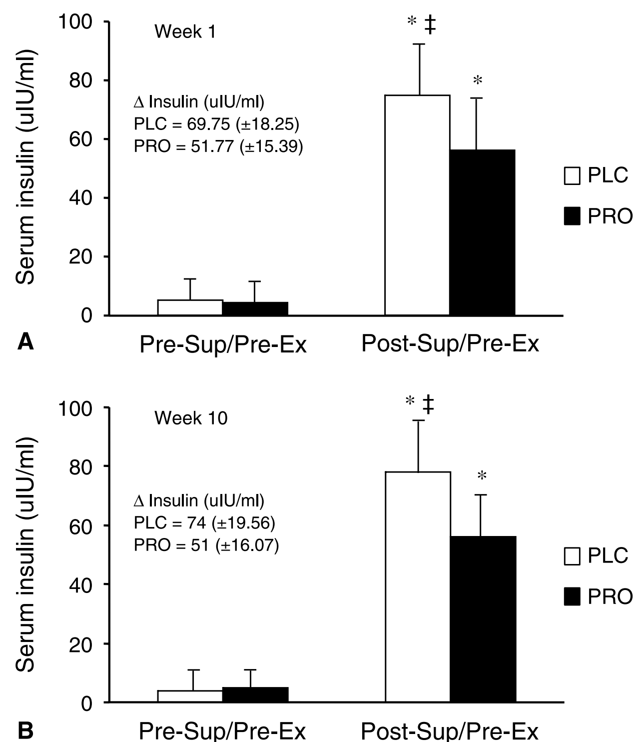

Fig. 1. A representation of the means (±SD) for **A** serum insulin levels 60 min after supplement ingestion prior to the first exercise session at wk 1, **B** serum insulin levels 60 min after supplement ingestion prior to the first exercise session at wk 10. Both supplements significantly increased serum insulin levels prior to exercise at wks 1 and 10; however, the response for CHO was greater than PRO ($p < 0.05$). *Significantly different from pre-training; †significantly different from CHO; ‡significantly different from PRO ($P \leq 0.05$)

were also observed for bench press ($p = 0.009$) and leg press strength ($p = 0.010$). Results showed 0.20 ± 0.08 and 0.61 ± 0.03 kg increases in relative bench press and leg press strength, respectively, for PLC; however, PRO underwent respective increases of 0.48 ± 0.02 and 1.13 ± 0.12 kg in relative bench press and leg press strength. These data indicate that both groups underwent significant increases in strength over the 10-wk period; however, the increases for PRO were greater than PLC (Table 3).

Myofibrillar protein content

No significant Group × Test interaction was observed ($p = 0.633$); however, significant main effects for Group ($p = 0.029$) and Test ($p = 0.006$) were observed and resulted in 10.54 ± 2.35 μg/mg increases for PLC, whereas PRO increased 25.03 ± 5.78 μg/mg. These data indicate that both groups underwent significant increases in myofibrillar protein over the 10-wks; however, the increases for PRO were greater than PLC (Table 3).

Serum insulin and IGF-1 levels

No significant Group × Test interaction was observed for insulin ($p = 0.468$); however, significant main effects for

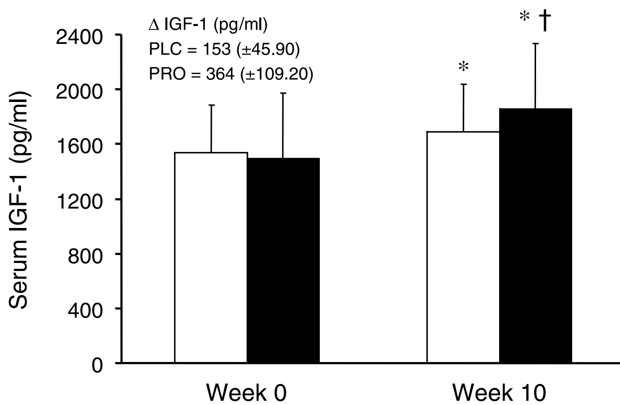

Fig. 2. Serum IGF-1 levels prior to supplement ingestion at week 1 and 24 h after the last exercise session at week 10. IGF-1 increased significantly in both groups; however, the increases for PRO were greater than those of CHO. *Significantly different from pre-training; †significantly different from CHO; ‡significantly different from PRO ($P \leq 0.05$)

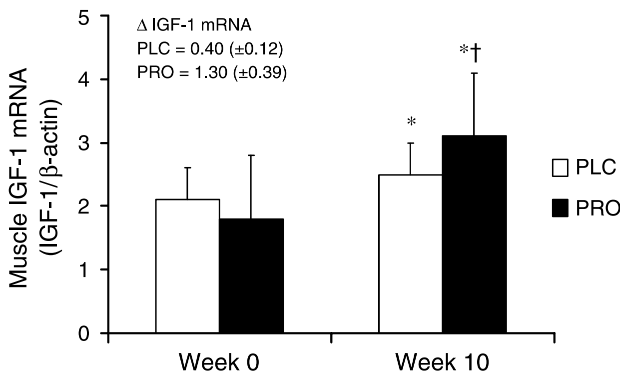

Fig. 3. Skeletal muscle IGF-1 mRNA expression is presented as ΔC_T values relative to β-actin. IGF-1 mRNA increased significantly in both groups; however, the increase for PRO was greater than those of CHO. *Significantly different from pre-training; †significantly different from CHO ($P \leq 0.05$)

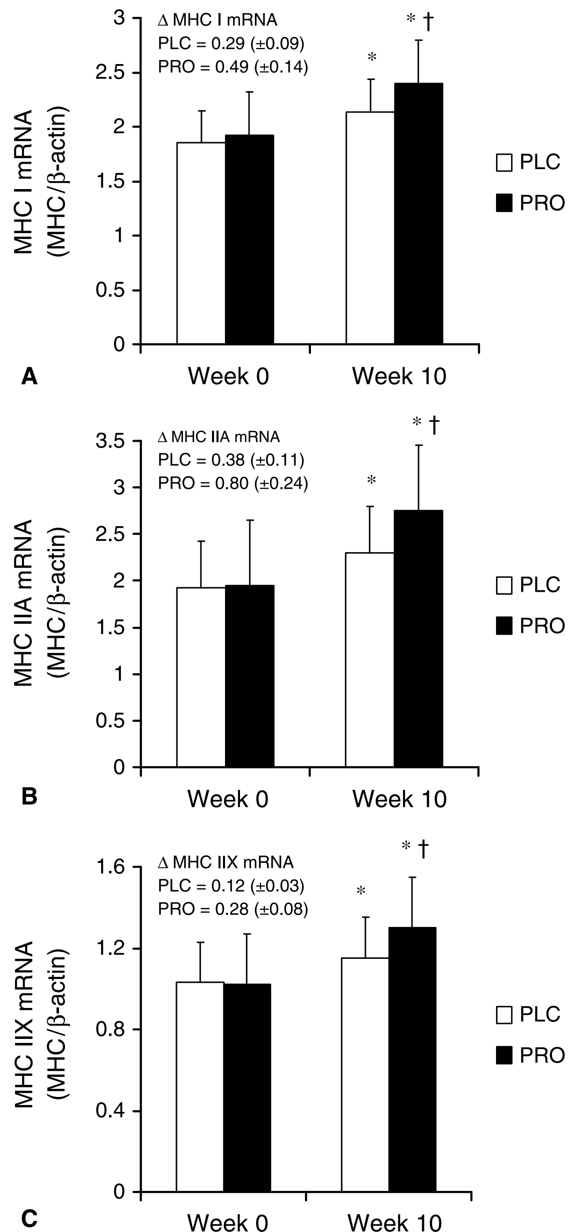

Fig. 4. Skeletal muscle MHC isoform mRNA expression is presented as ΔC_T values relative to β-actin for **A** MHC I, **B** MHC IIa, **C** MHC IIx. Both groups showed increases in the expression of MHC I, IIa, and IIx mRNA; however, the increases for PRO were greater than those of CHO. *Significantly different from pre-training; †significantly different from CHO ($P \leq 0.05$)

Group ($p = 0.012$) and Test ($p = 0.025$) were observed and resulted in respective increases of 69.75 ± 18.25 and 74.00 ± 19.56 uIU/ml at wks 1 and 10 for PLC, whereas PRO was increased 51.55 ± 15.39 and 51.00 ± 16.07 uIU/ml at wks 1 and 10, respectively. These data indicate that both supplements resulted in significant increases in serum insulin at wks 1 and 10; although, at both time points, the insulin response for PLC was greater than PRO (Fig. 1A and B).

For IGF-1, no significant Group × Test interaction was observed ($p = 0.872$); however, significant main effects for Group ($p = 0.029$) and Test ($p = 0.006$) were observed and resulted in a 153.00 ± 45.90 pg/ml increase for PLC, whereas PRO resulted in a 364.00 ± 109.20 pg/ml increase. These data indicate that both groups underwent significant increases in IGF-1 over the 10-wk period; however, the increases for PRO were greater than PLC (Fig. 2).

Skeletal muscle IGF-1 mRNA expression

No significant Group × Test interaction was observed ($p = 0.822$); however, significant main effects for Group ($p = 0.05$) and Test ($p = 0.029$) were observed for the relative mRNA expression of IGF-1 and resulted in 0.40 ± 0.12 increases in expression for PLC, whereas 1.30 ± 0.39 increases were observed for PRO. These data indicate that both groups underwent significant increases in IGF-1 mRNA expression over the 10-wk period; however, the increases for PRO were greater than PLC (Fig. 3).

Skeletal muscle MHC isoform mRNA expression

There were no significant Group × Test interactions for MHC I ($p = 0.154$), MHC IIa ($p = 0.073$), and MHC IIx ($p = 0.204$). Significant main effects for Group were located for MHC I ($p = 0.021$), MHC IIa ($p = 0.030$), and MHC IIx ($p = 0.01$). Significant main effects for Test were also observed for MHC I ($p = 0.041$), MHC IIa ($p = 0.034$), and MHC IIx ($p = 0.025$). The PLC group was shown to undergo relative expression increases of 0.29 ± 0.09, 0.38 ± 0.11, and 0.12 ± 0.03 for Types I, IIa, and IIx, respectively. However, PRO underwent even greater expression increases 0.49 ± 0.14, 0.80 ± 0.24, and 0.28 ± 0.08, respectively, for Types I, IIa, and IIx. These data indicate that both groups were expressing more of the MHC I, IIa, and IIx mRNA isoforms at the end of the 10-wk period; however, the increases for PRO were greater than PLC (Fig. 4A–C).

Skeletal muscle MHC isoform protein composition

There were no significant Group × Test interactions for MHC I ($p = 0.314$), MHC IIa ($p = 0.132$), and MHC IIx ($p = 0.342$). Significant main effects for Group were located for MHC I ($p = 0.025$), MHC IIa ($p = 0.013$), and MHC IIx ($p = 0.006$). In addition, significant main effects for Test were observed for MHC I ($p = 0.037$), MHC IIa ($p = 0.026$), and MHC IIx ($p = 0.007$). The PLC group was shown to undergo composition increases of 6.12 ± 0.28 and 5.74 ± 1.02% for Types I and IIa, respectively, and decreases of 12.14 ± 0.46% for Type IIx. Although, PRO underwent even greater increases of 8.84 ± 0.42 and

Table 4. Mean (±) and delta values for MHC protein isoform composition for the PLC and PRO groups prior to (wk 0) and after 10 wks of resistance training and supplementation

Variable/Session	PLC	PRO	Group	Test	Group × Test
% MHC Type I					
Wk 0	34.31 (2.62)	36.70 (2.25)			
Wk 10	40.43 (2.34)[a]	45.54 (2.67)[a,b]			
Δ MHC I	6.12 (0.28)	8.84 (0.42)	$p = 0.025$	$p = 0.041$	$p = 0.451$
% MHC Type IIa					
Wk 0	36.64 (3.16)	38.48 (2.67)			
Wk 10	42.38 (2.14)[a]	48.18 (1.47)[a,b]			
Δ MHC IIa	5.74 (1.02)	9.70 (1.57)	$p = 0.013$	$p = 0.026$	$p = 0.516$
% MHC Type IIx					
Wk 0	30.39 (2.73)	26.39 (2.59)			
Wk 10	18.25 (3.19)[a]	7.25 (3.14)[a,b]			
Δ MHC IIx	−12.14 (0.46)	−19.14 (0.55)	$p = 0.006$	$p = 0.007$	$p = 0.464$

Group = main effect for Group factor; Test = main effect for Test factor; Group × Test = interaction
[a] $p \leq 0.05$ from wk 0
[b] $p \leq 0.05$ from PLC

$9.70 \pm 1.57\%$, respectively, for Types I and IIa, while Type IIx decreased $19.14 \pm 0.55\%$. These data indicate that both groups underwent significant increases in MHC I and IIa, and decreases in MHC IIx, protein expression over the 10-wk period; however, the increases for PRO were greater than PLC (Table 4).

Discussion

We have herein demonstrated that a 10-wk heavy resistance-training program combined with the ingestion of a blend of whey and casein protein and free amino acids is more effective than an isocaloric carbohydrate placebo supplement in improving muscle strength and mass and markers indicative of muscle anabolism. It should be noted, however, that over the course of the study macronutrient intake within each group and between the two groups did not change significantly (Table 1), and both groups engaged in identical, supervised resistance training programs. Therefore, the greater improvements in muscle strength and mass seen in the PRO group are primarily the result of the ingestion of the protein and amino acid supplement compared to the ingestion of a carbohydrate placebo supplement.

As indicators of muscle performance and mass, we chose to evaluate muscle strength, thigh muscle mass, and body composition. Previous studies using varying protein supplement formulations and time points of ingestion have demonstrated that protein supplementation is more effective than carbohydrate at increasing muscle strength and performance and improving body composition. Ballard et al. (2005) showed that 12-wks of resistance training combined with 42 g of protein and 24 g of carbohydrate compared to a group ingesting 72 g of carbohydrate ingested immediately after the exercise session, and then later in the day, was more effective at increasing fat-free mass and decreasing fat mass than an isocaloric carbohydrate supplement. Walberg-Rankin et al. (2004) showed that a 10-wk resistance training combined with milk (0.92 g/kg carbohydrate, 0.21 g/kg protein, 0.06 g/kg fat) ingestion immediately following each exercise session was more effective at increasing muscle strength and fat-free mass when compared to a carbohydrate (1.25 g/kg carbohydrate) and electrolyte supplement. In addition, Bird et al. (2006) demonstrated that a 12-wk resistance-training program combined with either a 6% liquid carbohydrate and 6 g of essential amino acid (EAA) supplement ingested between sets of each exercise session was more effective than 6% carbohydrate in increasing muscle strength and fat-free mass.

The experimental protocol and protein supplement employed in the present study, however, more closely resembles the study by Andersen et al. (2005) who demonstrated that the ingestion of a protein supplement blend (16.6 g whey, 2.8 g casein, 2.8 g egg white, 2.8 g L-glutamine) 1 h before and after exercise combined with a 14-wk resistance-training program was more effective than an isocaloric carbohydrate in improving muscle function by way of vertical jump performance. In our study, we have specifically demonstrated that the 10-wk resistance-training program combined with the ingestion of a protein and amino acid supplement resulted in greater increases ($p < 0.05$) in bench press and leg press strength when compared to a carbohydrate placebo supplement (Table 3). Interestingly, these strength increases occurred concomitant with respective increases in total body mass, fat-free mass, and thigh mass for PLC and PRO; however, the increases for PRO were greater ($p < 0.05$) than those for PLC (Tables 2 and 3). Incidentally, there were no changes ($p > 0.05$) in total body water for either group (Table 2) that may have otherwise led to over-estimations in the evaluation of fat-free mass and the anthropometric measurements involved in determining thigh mass. Therefore, while our results show 10-wks of heavy resistance-training to be an effective stimulus at increasing muscle strength and mass, they also confirm that the ingestion of the protein and amino acid supplement to be even more effective than the carbohydrate placebo supplement.

We were also interested in evaluating systemic and local factors suggestive of skeletal muscle anabolism. Systemically we chose to evaluate serum insulin and IGF-1, while locally we chose skeletal muscle IGF-1 mRNA expression. As a result, we observed both supplements to be effective at increasing serum insulin (Fig. 1A and B); however, the protein and amino acid supplement was more effective than carbohydrate in increasing serum IGF-1 (Fig. 2) and muscle IGF-1 mRNA expression (Fig. 3). With insulin, it was our intent to compare the magnitude in which both supplements affected the levels of circulating insulin prior to engaging in an exercise session. Insulin receptor activation stimulates the PI3K-Akt/PKB-mTOR signaling pathway, which is known to have profound effects on the up-regulation of muscle-specific gene expression and protein synthesis (Bolster et al., 2004). Additionally, essential amino acids (leucine in particular) are known to up-regulate the activity of mTOR and p70S6 kinase, and hyper-phosphorylate 4E-BP1, suggesting that amino acids and insulin signaling do not function in isolation but both may function cooperatively to optimize the anabolic response in skeletal muscle

(Rasmussen et al., 2000). Our results show both supplements to significantly elevate serum insulin levels prior to the first and last exercise session of the study; however, the carbohydrate placebo elevated insulin more than the protein and amino acid supplement ($p < 0.05$).

Our results for insulin are supported by previous studies showing serum insulin levels to increase in response to consuming beverages containing 20 g casein and 20 g whey protein (Tipton et al., 2004), 6 g EAA and 40 g carbohydrate (Tipton et al., 1999), and 6% carbohydrate and 6 g EAA (Bird et al., 2006). Based on the observed insulin responses shown to occur with both supplements, and the availability of free amino acids in the protein and amino acid supplement, from our results it can therefore be assumed that up-regulations in insulin signaling were likely involved in up-regulating the down-stream anabolic responses in the synthesis of MHC and myofibrillar protein we observed to occur with both supplements, but more exacerbated in the protein and amino acid supplement (Tables 3 and 4).

Systemically, we showed serum IGF-1 to be elevated after training with PLC; however, PRO resulted in an increase in IGF-1 that was significantly greater ($p < 0.05$) than PLC (Fig. 2). In the aforementioned study by Ballard et al. (2005), a 12-wk resistance training program was shown to more effectively elevate serum IGF-1 levels in a group ingesting protein and carbohydrate compared to a group ingesting only carbohydrate. However, the other aforementioned study by Walberg-Rankin et al. (2004) found no differences in serum IGF-1 between groups ingesting either milk or carbohydrate after 10 wks of resistance training. IGF-1 is known to play an important role in the hypertrophic adaptation of muscle to resistance training, and IGF-1 increases muscle protein synthesis by way of its upstream regulation of the PI3K-Akt/PKB-mTOR signaling pathway (Sartorelli and Fulco, 2004). Therefore, the increased levels of insulin occurring from ingestion of the protein and amino acid supplement, along with the observed increases in IGF-1, may have potentiated the activity of the PI3K-Akt/PKB-mTOR signaling pathway, thereby leading to increases in muscle protein synthesis which could have subsequently resulted in the observed increases in muscle mass and strength over the course of the 10-wk period.

Skeletal muscle IGF-1 mRNA content has been shown to increase after heavy resistance exercise in humans (Bamman et al., 2001), and muscles undergoing hypertrophy have been shown to have increased levels of IGF-1 mRNA (Yang et al., 1996). While there are a number of studies showing increased IGF-1 mRNA in skeletal muscle after a single bout of resistance exercise (Hameed et al., 2003; Psilander et al., 2003), there are fewer studies investigating the effects of resistance training programs on IGF-1 mRNA expression. Hameed et al. (2003) demonstrated that 5-wks of resistance training significantly elevated IGF-1 mRNA expression in elderly men. We observed skeletal muscle IGF-1 mRNA expression to be increased in PLC, but even greater increases ($p < 0.05$) were observed in PRO (Fig. 3). However, the local expression of IGF-1 mRNA in skeletal muscle is thought to be load sensitive and acts independently of any change in serum IGF-1 (Adams, 1998). Although, the extent of this independence between serum IGF-1 and muscle IGF-1 mRNA in response to the ingestion of a protein and amino acid supplement during a resistance-training program is not known. Considering that the protein and amino acid supplement stimulated a greater amount of muscle mass and hypertrophy, the exacerbated expression we have demonstrated with IGF-1 mRNA is conceivable. However, there appears to be no studies available on resistance training and the subsequent effects of protein and amino acid supplementation on muscle IGF-1 mRNA expression.

As indicators of skeletal muscle protein synthesis, we were interested in evaluating the mRNA and protein expression of the MHC isoforms, along with changes in the content of myofibrillar protein. However, the data on MHC isoform expression and myofibrillar protein content resulting from protein and amino acid supplementation and resistance training appears to be non-existent. The aforementioned study by Andersen et al. (2005) demonstrated that 14 wks of resistance training combined a group ingesting a blend of whey, casein, and egg protein ingested 1 h before and after each training session displayed increases in Types I and II muscle fiber hypertrophy compared to a carbohydrate group. In the present study, we evaluated pre-translational activity of MHC synthesis by evaluating MHC isoform mRNA expression. The PLC group was shown to undergo increases for Types I, IIa, and IIx; however, PRO underwent even greater increases ($p < 0.05$) for Types I, IIa, and IIx (Fig. 4A–C).

Additionally, we also sought to evaluate the post-translational synthesis of MHC by evaluating changes in the composition of MHC isoform protein. The PLC group was shown to undergo increases in Types I and IIa, and a decrease in Type IIx ($p < 0.05$). Although, PRO underwent even greater increases for Types I and IIa, while Type IIx decreased ($p < 0.05$) (Table 4). We then chose to evaluate myofibrillar protein content as an overall indicator of muscle-protein synthesis and observed myofibrillar protein to be increased in PLC, whereas PRO increased more than PLC ($p < 0.05$) (Table 3).

While not using a protein and amino acid supplement, data from the carbohydrate placebo group from our previous study conducted with a similar resistance training protocol lends support to the present results in that 12-wks of heavy resistance training combined with 20 g/d of carbohydrate supplementation was more effective at increasing MHC expression and myofibrillar protein content than in a resistance-trained control group without carbohydrate (Willoughby and Rosene, 2001). In our previous study, we showed similar changes in MHC mRNA and protein expression and myofibrillar protein content as we have presented herein, confirming that 10–12 wks of heavy resistance training is indeed capable of up-regulating the mechanisms involved in increasing the synthesis of MHC and other myofibrillar proteins.

Increased muscle protein synthesis presumably results from enhanced transcription, enhanced mRNA stability, an elevated translation rate, or a combination of the three (Psilander et al., 2003). A resistance exercise study in humans has indicated that MHC protein expression can be up-regulated without a concomitant increase in mRNA expression (Welle et al., 1999). However, we have recently shown that a single bout of heavy resistance exercise in humans resulted in an up-regulation in MHC isoform and myogenic regulatory factor mRNA, along with an increase in the protein expression of the myogenic regulator factors Myo-D and myogenin (Willoughby and Nelson, 2002). Therefore, it appears that resistance training involves both transcriptional and translational alterations in muscle than can be further augmented with the ingestion of a protein and amino acid supplement. As such, changes in translation may be the basic regulatory mechanism in hypertrophy. Transcription of specific muscle-specific genes, such as the myogenic regulatory transcription factors Myo-D and myogenin or growth factors such as IGF-1, may provide a secondary role in modifying the rate of muscle protein synthesis by providing mRNA template for translational activity (Psilander et al., 2003).

Since we observed significant increases in serum insulin prior to the first and last exercise sessions, it is not unreasonable to assume that serum insulin was consistently elevated prior to and after each exercise session once the supplements were ingested. In addition, the protein and amino acid supplement was shown to have a greater effect on serum IGF-1 and muscle IGF-1 mRNA expression. Based on the fact that the participants' dietary intake of macronutrients did not change, and that all participants continued to train with the same exercises and relative intensity, the consistent elevation in pre- and post-exercise serum insulin and IGF-1 likely up-regulated the aforementioned insulin and IGF-1 signaling pathways, and were involved in increasing muscle anabolism and hypertrophy that subsequently translated into the increases we observed in thigh mass, fat-free mass, total body mass, and muscle strength. The fact that the protein and amino acid supplement was shown to have a more pronounced effect when compared to a carbohydrate placebo, despite a less pronounced serum insulin response, may have been due to the added free amino acids, particularly 6 g leucine, where cooperativity with insulin and IGF-1 signaling may have exacerbated muscle protein synthesis. As a result, we conclude that 10-wks of heavy resistance-training combined with the timed ingestion of a supplement containing a 40 g blend of whey and casein and free amino acids is more effective than 40 g of carbohydrate placebo in increasing muscle strength and mass, body composition, and increasing systemic and local indicators suggestive of skeletal muscle anabolism and hypertrophy.

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