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Co-ingestion of carbohydrate with branched-chain amino acids or L-leucine does not preferentially increase serum IGF-1 and expression of myogenic-related genes in response to a single bout of resistance exercise

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Co-Ingestion of Carbohydrate with Branched Chain Amino Acids or L-Leucine Does Not Preferentially Increase Serum IGF-1 and Expression of Myogenic-Related Genes in Response to a Single Bout of Resistance Exercise

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Abstract This study determined if the co-ingestion of carbohydrate (CHO) with branched-chain amino acids (BCAA) or L-leucine (LEU) preferentially affected serum IGF-1 and the expression of myogenic-related genes in response to resistance exercise (RE). Forty one, college-age males were randomly assigned to 1 of 4 groups: CHO, CHO-BCAA, CHO-LEU, or placebo (PLC). Resistance exercise consisted of 4 sets of leg press and leg extension at 80% 1RM. Supplements were ingested peri-exercise, and venous blood and muscle biopsies were obtained pre-exercise (PRE), and at 30, 120, and 360 min post-exercise. Serum IGF-1 was determined with ELISA, and skeletal muscle mRNA expression of myostatin, ActRIIB, p21kip, p27kip, CDK2, cyclin B1, cyclin D1, Myo-D, myogenin, MRF-4, and myf5 was determined using real-time PCR. Results were determined with two-way ANOVA for serum IGF-1 and two-way MANOVA for mRNA expression. Serum IGF-1 in CHO and CHO+BCAA was greater than PLC ($p < 0.05$) but was not affected by RE ($p > 0.05$). Significant differences were detected between groups for myostatin, ActIIB, MyoD, and myf5 mRNA expression showing CHO to be significantly different than CHO+BCAA, CHO+LEU, and PLC ($p < 0.05$). At 30, 120 and 360 min post-exercise, p21cip was significantly less than PRE, whereas cyclin D1 was greater than PRE at 120 and 360 min post-exercise ($p < 0.05$). The co-ingestion of CHO with either BCAA or L-leucine in conjunction with RE had no preferential effect on serum IGF-1 or pre-translational markers indicative of myogenesis.

Keywords gene expression, satellite cell, hypertrophy, protein synthesis

INTRODUCTION

Skeletal muscle exhibits inherent plasticity in its ability to adapt to various mechanical, hormonal, and nutritional stimuli by possessing the capability to alter its phenotype. Moreover, the ability of skeletal muscle to undergo hypertrophic changes in respone to resistance exercise is under the control of anabolic and catabolic signaling pathways that operate at both the pre- and post-translational levels to help maintain the balance between protein synthesis and proteolysis. Two primary cellular processes are involved with hypertrophy responses in mammalian skeletal muscle. The first process involves both pre- and post-translational mechanisms that result in the accretion of myofibrillar protein and subsequent myofiber enlargement due to increases in the myonuclear domain (Bickel et al. 2005; Shelmadine et al. 2009). The second process results in myofiber enlargenment due to a decreased myonuclear domain resulting from activation and proliferation of myogenic satellite cells which fuse into muscle fibers, thereby providing additional myonuclei for augmented transcriptional activity and subsequent protein synthesis (Han et al. 2008). Muscle damage that occurs in response to resistance exercise from both dynamic/isotonic (Willoughby & Nelson, 2002; Psilander et al.

2003) and forced-lengthening (eccentric) muscle contractions (Farup et al. 2014) have been shown to effectively increase markers of satellite cells activity and/or the number of activated satellite cells.

Muscle protein accretion, mediated by satellite cell activation, is modulated by the motiotic and myogenic activity of insulin-like growth factor 1 (IGF-1), which functions in an endocrine/autocrine/paracrine mode and functions through the binding to the IGF-1 receptor (IGF-1R). This ligand-receptor interaction triggers intracellular signaling cascades through extracellular-regulated kinase (ERK) and protein kinase B (Akt) which play a role in the proliferation and differentiation, respectively, of satellite cells (Philippou et al. 2007). Upon activation, however, the process of myogenesis is controlled by several myogenic regulatory factors (MRFs) which serve as transcription factors and act as terminal effectors of the signaling cascades and produce appropriate stage-specific transcripts. The expansion of activated satellite cells occurs from myogenic factor 5 (myf5). Myoblast determination protein (Myo-D) is involved in determining the differentiation poteintial of an activated myoblast and acts together with myogenin to drive differentiation, whereas muscle-specific regulatory factor 4 (MRF-4) is required for hypertrophy as it plays a role in the maturation of myotubes (Knight & Kothary, 2011).

Along with the MRFs, the myogenic activation of satellite cells is also regulated by cyclin-dependent kinases (CDKs) which have a catalytic dependence on the cyclin family of regulatory proteins. Cyclin D, a G_1 cyclin which regulates progression through G_1 and entry into the S phase of the cell cycle, is responsible for its role in myoblast differentiation by activating CDK2 (Knight & Kothary, 2011). For myoblasts to differentiate, the cell cyle must be exited and the restraints the CDKs place on differentitation must be removed. As such, the expression of cyclin D1, cyclin B1, and CDK2 decreases with differentiation (Jahn et al. 1994; Skapek et al. 1995).

Concomitant with the decreased expression of cyclin/CDKs, there is an increase in the levels of the cyclin-dependent kinase-interacting protein/kinase-inhibitory protein family (CIP/KIP). The CIP/KIPs, p21cip and p27kip, inhibit all G1 CDKs (Knight & Kothary, 2011). Furthermore, myostatin which is a member of the transforming growth factor beta (TGF-β) family works in a paracrine/autocrine manner through the activin IIB receptor (ACTRIIB), and is a negative regulator of myogenesis and subsequent muscle protein accretion by repressing the expression of Myo-D, myogenin, myf5, and p21cip, and by down-regulating cyclin D1 during the G_1 phase of the cell cycle (Langley et al. 2002).

Nutrients, amino acids in particular, play an important role in regulating muscle protein accretion. Moreover, delivery and uptake of carbohydrate (CHO) and protein may influence satellite cell activity, as it has recently been shown that both nutrients increased the number of satellite cells per myonuclei during recovery following eccentric exercise (Farup et al. 2014). The mechanism(s) in which nutrient availability may increase myogenic activity is not well known but may be associated with the process in which CHO and branched-chain amino acids (BCAAs) contained in protein are able to increase muscle protein synthesis (Miller et al. 2003) and upregulate intramuscular signaling cascades known to effect myogenic regulators such as ERK (Di Camillo et al. 2014) and Akt (Ferreira et al. 2014). Relative to the role that nutrient availability plays in inducing muscle hypertrophy, it has been shown that the immediate ingestion of either CHO and/or amino acids after resistance exercise results in an increase in skeletal muscle protein synthesis (Biolo et al. 1997; Miller et al. 2003). The branched-chain amino acids (BCAAs), in particular leucine, simulate protein synthesis through signalling pathways involving mTOR (mammalian target of rapamycin) (Kimball & Jefferson, 2006; Rennie, 2007) in a dose-response manner and seems independent of serum insulin and IGF-1. Furthermore, we have previously shown that the insulinogenic response from the co-ingestion of CHO and BCAA instigated no related response to RE in mediating increases in the activity of Akt, mTOR, and

P70S6K, signaling markers indicative of muscle protein synthesis (Ferreira et al. 2014).

However, currently there appear to be no published data available to elucidate the effects that CHO and BCAAs may have on the gene expression of myogenic markers on satellite cell activation in response to resistance exercise in humans. Therefore, the purpose of the study was to determine the effects of the co-ingestion of CHO with either BCAA or LEU in response to a single bout of heavy exercise on serum IGF-1 and the skeletal muscle expression of the myogenically-related genes, myostatin, ActRIIB, p21cip, p27kip, CDK2, cyclin B1, cyclin D1, MyoD, myogenin, MRF-4, and myf5.

METHODS

Experimental Design

The study involved a fully-randomized, four-group, factorial, repeated-measures design in which each group received a separate peri-exercise nutritional supplement while performing an identical bout of resistance exercise. Blood and muscle samples were obtained immediately before and 30, 120, and 360 min following exercise. These data presented herein are a subset from our previous study in which a more comprehensive discussion of the methods, illustration of study timeline, and various data, such as serum insulin and dietary intake, is presented elsewhere (Ferreira et al. 2014).

Participants

A total of 46 individuals were screened for eligibility in the study. Five individuals were not included in the study for the following reasons: two did not meet inclusion criteria, one chose not to participate due to the muscle biopsies, and two chose not to participant due to time constraints. Therefore, 41 physically-active, apparently-healthy men between the ages of 18-30 years served as participants in the study. While the men were physically active (engaging in non-structured activity of a physical nature), they were not resistance-trained. Participants were excluded if they had been performing a structured resistance training program more than thrice weekly in the previous year and/or who had performed lower-body resistance exercise in the 14 days immediately prior to the study. Only participants considered as either low or moderate risk for cardiovascular disease and with no contraindications to exercise as outlined by the American College of Sports Medicine (Whaley, 2005) and/or who had not consumed any nutritional supplements (excluding multi-vitamins and protein supplements) one month prior to the study were allowed to participate. All 41 participants who began were able to successfully completed the study without any complications.

Familiarization Session, Baseline Strength Testing, and Randomization to Groups

During the familiarization session, participants received both written and verbal description of the study design and requirements and read and signed university-approved documents granted by the Institutional Review Board for Human Subjects of Baylor University. Height and body mass measures were obtained, then participants had their one repetition maximum (1-RM) determined on the angled leg press (Nebula Fitness, Inc., Versailles, OH) and knee extension (Body Masters, Inc., Rayne, LA) exercises as previously described (Ferreira et al. 2014). After baseline strength testing, each subject was matched according to muscle strength and body mass and then randomly assigned to one of four supplement groups [CHO (n = 10), CHO + BCAA (n = 10), CHO + LEU (n = 11), placebo (n = 10)].

Resistance Exercise Session Protocol

Participants arrived at the lab in the morning between 8-10 am following an overnight fast, adequately hydrated, and rested (no lower-body exercise for 72 hours prior to baseline testing). Participants brought a completed 48-hr food record to the resistance exercise session. Participants performed four sets each of the angled leg press and knee extension exercises at 80% 1-RM until failure (approximately 8-12 repetitions). Rest periods were 2.5 min between both sets and exercises constituted a 1:5 work to rest ratio. The

exercise session was approximately 30 min in duration (Ferreira et al. 2014).

Supplementation Protocol

The BCAA supplement (Now Foods, Bloomingdale, IL) was comprised of 50% leucine, 25% isoleucine, and 25% valine. The orally ingested dosage of BCAA was 120 mg/kg body mass (60 mg/kg leucine, 30 mg/kg isoleucine, and 30 mg/kg valine per each participant) (Karlsson et al. 2004). The LEU supplement (Source Naturals, Scotts Valley, CA) was provided at a dosage of 120 mg/kg per each participant. The amount of maltodextrin in the CHO supplement for each group was provided at a dosage of 1.5 g/kg body mass (approximately 120 g per each participant), which had been shown to induce a significant insulogenic response (Schumm et al. 2008). The placebo (PLC) supplement was non-caloric Crystal Light®. Each supplement was ingested in equal parts dissolved in 150 mL of a flavored non-caloric beverage (of Crystal Light®), for a total supplement ingestion of 450mL. Participants ingested the assigned supplement dose at each of the following time points: 30 min prior to RE, immediately prior to RE, 5 min following RE.

Venous Blood Sampling and Muscle Biopsies

Venous blood was collected from the antecubital vein using a vacutainer apparatus and standard phlebotomy procedures. Vacutainer tubes were immediately centrifuged at 1,100 g for 15 minutes (Cole Parmer, Vernon Hills, IL). Serum was then separated and transferred in sterile cryo-vials and stored at -80°C for future analyses.

Upon subcutaneous anesthetization with 1% lidocaine, muscle biopsies (~50-75 mg) were extracted from the left vastus lateralis midway between the patella and the greater trochanter at a depth of 1-2 cm using the percutaneous biopsy technique with suction as previously described (Bergstrom, 1975; Evans et al. 1982; Ferreira et al., 2014). The same incision location was used for the subsequent excisions, and sampling depth was verified by depth markings on the biopsy needle. Following the initial biopsy, for each subsequent biopsy the needle was rotated in the clock-wise direction from the previous biopsy (biopsy 1 = 1,200 hours; biopsy 2 = 1,500 hours; biopsy 3 = 1,800 hours; biopsy 4 = 2,100 hours) so the sample was taken from within the same area of muscle. Previous research has shown that a multiple biopsy technique does not alter exercise-induced gene expression (Lundby et al. 2005; Willoughby & Nelson, 2002). Collected muscle tissue samples were placed in sterile cryovials, flash frozen in liquid nitrogen, and stored at -80° C for future analysis. Blood and muscle samples were obtained immediately prior (PRE) to the first bolus of supplement, and at 30, 120, and 360 min following RE.

Serum IGF-1

Serum IGF-1 was analyzed using a commercially available ELISA kit (Diagnostic Systems Laboratories Inc. Webster, TX). According to the manufacturer, the sensitivity for this assay was 0.01ng/mL. A standard curve was generated using a control peptide and absorbances were determined in duplicate at an optical density of 450 nm with a microplate reader (Wallac Victor 1420, Perkin Boston, MA, USA). IGF-1 concentrations were quantified using MicroWin microplate data-reduction software (Mikrotek Laborysteme, Germany). The overall intra-assay coefficient of variation was 7.1%.

Skeletal Muscle Total RNA Isolation and Quantitation and cDNA Synthesis

Following our previously established procedures (Wilborn et al. 2009; Willougbhy et al. 2007), approximately 20 mg of muscle tissue was homogenized in a of monophasic solution of phenol and guanidine isothiocyanate contained within the TRI-reagent (Sigma Chemical Co., St. Louis, MO). Total RNA concentrations were determined spectrophotometerically (Helio γ, Thermo Electron, Milford, MA) by optical density (OD) at 260 nm using an OD260 equivalent to 40 μg/μl (Ausubel et al. 2002), and the final concentration expressed relative to muscle wet weight. Aliquots of total RNA were separated with 1% agarose gel electrophoresis, ethidium bromide

stained, 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, as well as an OD260/OD280 ratio of approximately 2.0. Test-retest reliability of performing this procedure of total RNA integrity on samples in our laboratory has demonstrated low mean coefficients of variation and high reliability (1.8%, intraclass r = 0.96). The RNA samples were stored at -80°C until later analysis.

Total skeletal muscle RNA was reverse-transcribed to synthesize cDNA using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). The cDNA concentration was determined by using an OD260 equivalent to 50 µg/µl (Ausubel et al. 2002), and the starting cDNA template concentration was standardized by adjusting all samples to 200 ng prior to PCR amplification (Wilborn et al. 2009; Willoughby et al. 2007).

Oligonucleotide Primers for PCR

The mRNA sequences of human skeletal muscle published in the NCBI Entrez Nucleotide database (www.ncdi.nlm.hih.gov) were used to construct oligonucleotide PCR primers with Beacon Designer software (Bio-Rad, Hercules, CA, USA) (Table 1). Primer pairs were synthesized commercially (Integrated DNA Technologies, Coralville, IA). β-actin was used as an external control standard for each reaction 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 in human skeletal muscle following acute exercise (Mahoney et al. 2004; Wilborn et al. 2009; Willoughby et al. 2007).

Table 1. Nucleotide sequences of sense and antisense primers used for real-time PCR.

Primer Name	NCBI Accession Number	Sense Sequence (5' → 3')	Anti-Sense Sequence (5' → 3')	Amplicon Size (bp)
β-Actin	NM_001101	ATCGTGCGT GACATTAAG	GTCATCACC ATTGGCAAT	102
Myostatin	NM_005259	CAAGAAYAGAAG CCATTAAGATAC	CGTTGTAGCG TGATAATCG	194
ActRIIB	NM_001106	GCCTTGCCAT CAGATTGTG	GCCATCAGAAC CAGATATACC	155
Cyclin B1	NM_031966	TGCCTCTCCA AGCCCAATG	TTCCTCAAGTTGT CTCAGATAAGC	178
Cyclin D1	NM_001758	CAACTTCCTG TCCTACTACC	TCCTCCTCC TCCTCTTCC	177
CDK2	X61622	GCACTACGAC CCTAACAAG	CCACCTGAGT CCAAATAGC	190
P21cip	L25610	TCCAGCGACCT TCCTCATCCAC	TCCATAGCCTCT ACTGCCACCATC	108
p27kip	NM_004064	CAGGAGAGC CAGGATGTC	TAGAAGAATC GTCGGTTGC	175
Myo-D	X56677	CGCCACCGC CAGGATATG	GTCATAGAAGT CGTCCGTTGTG	108
Myogenin	X62155	TGAGGAGGTAAC ATAGAAGGACAG	CAGCAGCCG TGGTCAGAG	148
MRF-4	NM_002469	ACTGGCTCCTAT TTCTTCTACTTG	TTCCTCTCC GCTGCTGTC	159
myf5	X14894	GAGCAGGTG GAGAACTAC	GTGGCATATACA TTTGATACATC	170

Real-Time PCR Amplification and Quantitation

Following our previous procedures (Wilborn et al., 2009; Willoughby et al. 2007), each reaction was amplified using real-time quantitative PCR system (iCycler IQ Real-Time PCR Detection System, Bio-Rad, Hercules, CA, USA). To help control for differences in amplification efficiency during thermocycling, all PCR reactions were prepared from the same stock solution. The specificity of the PCR was demonstrated with an absolute negative control using a separate PCR reaction containing no cDNA and a single gene product was confirmed for each primer pair using DNA melt curve analysis. Additionally, to assess positive amplification of mRNA and correct amplicon size, random aliquots of PCR reaction mixtures from each sample were agarose gel electrophoresed stained with ethidium bromide and ultra-violetly illuminated (Chemi-Doc XRS, Bio-Rad, Hercules, CA). Based on our previous work, the relative expression of mRNA was determined as the ratio of the threshold cycle (Ct) values of each target mRNA to the corresponding Ct values of β-actin for each muscle sample (Serra et al. 2012; Wilborn et al., 2009; Willoughby et al. 2007). Test-retest reliability of performing this procedure of mRNA expression on samples in our laboratory has demonstrated low mean coefficients of variation and high reliability (1.6%, intraclass r = 0.95).

Statistical Analyses

A one-way analysis of variance (ANOVA) was calculated on all dependent variables to determine if significant differences existed at baseline between the four groups. A one-way ANOVA was performed on exercise volume for the four groups. A 4 × 4 (group × time point) univariate ANOVA was performed for serum IGF-1. A 4 × 4 (group × time point) multivariate ANOVA (MANOVA) was determined on skeletal muscle mRNA variables. Due to the likelihood that the expression of our targeted genes were possibly being contingent on one another, the use of a MANOVA analysis prevented the chance of committing a Type I error that could result from the use of repeated univariate ANOVA; in contrast, this would increase the likelihood of performing type II error, particularly with a low sample size. Separate ANOVAs on each dependent variable were conducted as follow-up tests to the MANOVA. To control for alpha-level inflation, the Bonferroni test was utilized for multiple comparisons.

In addition to reporting probability values, an index of effect size was reported to reflect the magnitude of the observed effect. The index of effect size utilized was partial Eta squared (η2), which estimates the proportion of variance in the dependent variable that can be explained by the independent variable. Partial Eta squared effects sizes were determined to be: weak = 0.17, medium = 0.24, strong = 0.51, very strong => 0.70 as previously described by (O'Connor et al., 2007). In addition, for all statistical analyses not meeting the sphericity assumption for the within-subjects analyses, a Greenhouse-Geisser correction factor was applied to the degrees of freedom in order to increase the critical F-value to a level that would prevent the likelihood of committing a type I error. All statistical procedures were performed using SPSS 21.0 software (Chicago, IL) and a probability level of <0.05 was adopted throughout.

RESULTS

Participant Demographics

Descriptive statistics (means ±SD) for body mass, age, and height, are reported in Table 2. No significant differences were found to exist at baseline between groups for participant demographics, serum IGF-1, or any of the mRNA variables assessed ($p > 0.05$).

Total Lifting Volume and Macronutrient Intake

The mean ±SD exercise volume, expressed as total lifting volume for both the leg press and leg extension for the resistance exercise sessions was 16,962 ±7,699 kg, 16,298 ±3,608 kg, 15,692 ±4,516 kg, and 17,784 ±6,600 kg for CHO,

CHO+BCAA, CHO+LEU, and PLC, respectively. Results demonstrated no significant differences in exercise volume ($p > 0.05$). Data from nutritional intake is presented elsewhere (Ferreira et al. 2014), but was not significantly different between groups ($p > 0.05$).

Serum IGF-1

For serum IGF-1, results revealed no significant group × time interaction ($p = 0.99$; effects size = 0.019) or main effect for time ($p = 0.97$; effect size = 0.004). However, a significant main effect for group was found ($p = 0.001$; effect size = 0.135). Post-hoc analysis showed that CHO and CHO+BCAA were significant greater than PLC (Table 3).

Skeletal Muscle mRNA Expression

For the comprehensive multivariate analysis of all mRNA variables, Wilks' Lambda produced a significant group × time interaction ($p = 0.001$; effect size = 1.00). There were also significant multivariate differences for the main effects for group ($p = 0.001$; effect size = 1.00) and time ($p = 0.001$; effect size = 1.00).

Table 2. Participant Demographics.

Demographics	CHO	CHO+BCAA	CHO+LEU	PLC	$p \leq 0.05$
Height (cm)	183.3 ±6.5	178.0 ±5.4	178.1 ±8.4	178.1 ±9.0	0.32
Body Mass (kg)	81.4 ±14.0	77.3 ±15.3	81.8 ±16.9	83.0 ±11.2	0.83
Age (yrs)	20.4 ±2.5	21.8 ±2.6	20.7 ±3.2	21.0 ±2.4	0.69

Data are reported as means ±SD. No significant differences were found between groups at baseline relative to participant demographics ($p > 0.05$).

Table 3. Serum IGF-1 Levels for the CHO, CHO+BCAA, CHO+LEU, and PLC Groups.

Variable	Pre	30 Min Post-Ex	120 Min Post-Ex	360 Min Post-Ex	G	T	G×T
IGF-1 (ng/ml)					**0.001***	0.97	0.99
CHO	337.35 ±112.49	305.01 ±90.09	327.78 ±87.03	338.49 ±91.42	**> PLC**		
CHO+BCAA	345.89 ±91.70	397.41 ±141.34	360.82 ±81.07	349.52 ±89.33	**> PLC**		
CHO+LEU	302.95 ±115.37	303.05 ±92.27	275.09 ±72.79	270.01 ±67.59			
PLC	257.61 ±69.90	275.62 ±88.85	267.68 ±87.28	267.16 ±84.92			

Data are presented as means ±SD for the serum values of IGF-1. G, T, and GxT represent the univariate main effects for group, time, and group x time interaction, respectively, at a probability level of ≤ 0.05. The symbol * denotes a significant difference for the group main effect. CHO and CHO+BCAA were significantly greater than PLC.

mRNA Expression for Markers of Myogenic Activation

Upon further analysis with univariate ANOVA, a significant group × time interaction was observed for cyclin D1 ($p = 0.001$; effect size = 1.00). Post-hoc analysis showed that PLC was significantly greater than CHO, CHO+BCAA, and CHO+LEU at 30, 120, and 360 min post-exercise. Significant group main effects were observed for CDK2 ($p = 0.002$, effect size = 0.92), MyoD ($p = 0.006$; effect size = 0.86), and myf5 ($p = 0.007$; effect size 0.85). For cyclin B1, a strong trend towards significance was observed base on a p-value of 0.06 and an effect size of 0.60. Post-hoc analysis showed that for CDK2, CHO+BCAA, CHO+LEU, and PLC were significantly greater than CHO. In regard to Myo-D, CHO, CHO+LEU, and PLC were greater than CHO+BCAA. For myf5, CHO and CHO+BCAA were significantly greater than PLC and CHO+LEU.

Analysis of the time main effects also produced significant differences between time points for cyclin B1 ($p = 0.001$; effect size = 0.986). Post-hoc analysis revealed that PRE was significantly less than 30 and 120 min post-exercise. For MRF-4, a strong trend towards significance was observed base on a p-value of 0.07 and an effect size of 0.58. (Table 4).

Table 4. mRNA Expression for Markers of Myogenic Activation for the CHO, CHO+BCAA, CHO+LEU, and PLC Groups.

Variable	Pre	30 Min Post-Ex	120 Min Post-Ex	360 Min Post-Ex	G	T	G×T
CDK2					**0.002 ***	0.88	0.76
CHO	0.98 ±0.04	1.01 ±0.04	1.01 ±0.03	0.99 ±0.05			
CHO+BCAA	1.06 ±0.05	1.05 ±0.06	1.03 ±0.07	1.04 ±0.04	**> CHO**		
CHO+LEU	1.04 ±0.06	1.02 ±0.07	1.01 ±0.06	1.05 ±0.06	**> CHO**		
PLC	1.05 ±0.08	1.02 ±0.06	1.05 ±0.04	1.03 ±0.07	**> CHO**		
Cyclin B1		**> PRE**	**> PRE**		0.06	**0.001 †**	0.13
CHO	1.04 ±0.02	1.01 ±0.03	1.02 ±0.01	1.02 ±0.02			
CHO+BCAA	1.03 ±0.02	1.02 ±0.01	1.07 ±0.06	1.04 ±0.04			
CHO+LEU	1.04 ±0.05	1.01 ±0.02	1.04 ±0.03	1.05 ±0.04			
PLC	1.02 ±0.05	1.01 ±0.01	1.05 ±0.02	1.02 ±0.03			
Cyclin D1			**> PRE, 30 MIN POST-EX**	**> PRE, 30 MIN POST-EX**	**0.001 ***	**0.001 †**	**0.001 ‡**
CHO	0.93 ±0.04	0.99 ±0.03	0.98 ±0.02	1.04 ±0.02	**< PLC**		
CHO+BCAA	1.00 ±0.04	0.98 ±0.02	1.01 ±0.03	1.00 ±0.01	**< PLC**		
CHO+LEU	0.98 ±0.03	0.95 ±0.02	1.01 ±0.01	1.03 ±0.03	**< PLC**		
PLC	0.99 ±0.03	1.01 ±0.03	1.04 ±0.02	1.06 ±0.04			
Myo-D					**0.006 ***	0.52	0.79
CHO	1.07 ±0.09	1.04 ±0.07	1.05 ±0.08	1.05 ±0.07	**> CHO+BCAA**		
CHO+BCAA	1.00 ±0.05	1.02 ±0.09	0.97 ±0.07	0.99 ±0.06			
CHO+LEU	1.04 ±0.07	1.04 ±0.12	1.07 ±0.07	1.03 ±0.08	**> CHO+BCAA**		
PLC	1.07 ±0.06	1.02 ±0.09	1.02 ±0.06	1.01 ±0.05	**> CHO+BCAA**		
Myogenin					0.34	0.58	0.54
CHO	1.12 ±0.10	1.11 ±0.06	1.09 ±0.10	1.11 ±0.11			
CHO+BCAA	1.08 ±0.09	1.08 ±0.10	1.09 ±0.12	1.07 ±0.10			
CHO+LEU	1.05 ±0.04	1.13 ±0.11	1.10 ±0.07	1.06 ±0.11			
PLC	1.21 ±0.06	1.14 ±0.07	1.06 ±0.08	1.10 ±0.04			
MRF-4					0.13	0.07	0.62
CHO	1.20 ±0.15	1.19 ±0.15	1.19 ±0.18	1.20 ±0.17			
CHO+BCAA	1.20 ±0.12	1.18 ±0.11	1.20 ±0.14	1.16 ±0.12			
CHO+LEU	1.28 ±0.10	1.24 ±0.19	1.27 ±0.15	1.19 ±0.12			
PLC	1.03 ±0.10	1.31 ±0.12	1.23 ±0.13	1.12 ±0.12			
Myf5					**0.007 ***	0.26	0.30
CHO	1.06 ±0.09	1.02 ±0.07	1.02 ±0.08	1.02 ±0.09	**> PLC, CHO+LEU**		
CHO+BCAA	1.01 ±0.05	1.03 ±0.07	1.04 ±0.09	1.00 ±0.06	**> PLC, CHO+LEU**		
CHO+LEU	1.08 ±0.05	1.06 ±0.07	1.07 ±0.05	1.01 ±0.07			
PLC	1.09 ±0.05	1.07 ±0.06	1.04 ±0.03	1.09 ±0.06			

Data are presented as means ±SD for the ratio of Ct values for β-actin and each target mRNA variable. G, T, and G×T represent the univariate main effects for group, time, and group × time interaction, respectively, at a probability level of ≤ 0.05. The symbol * denotes a significant main effect for group. For CDK2, CHO+BCAA, CHO+LEU, and PLC were significantly greater than CHO. For cyclin D1, PLC was significantly different from CHO, CHO+BCAA, and CHO+LEU. For MyoD, CHO+BCAA was significant different than CHO, CHO+LEU, and PLC. For myf5 CHO and CHO+BCAA were significantly different from PLC, and CHO+LEU was significantly different from CHO+BCAA. The symbol † denotes a significant main effect for test. For cyclin B1, PRE was significantly less than 30, 120, and 360 min post-exercise. The symbol ‡ denotes a significant group x time interaction. PLC was greater than CHO, CHO+BCAA, and CHO+LEU at 120 and 360 min post-exercise.

Table 5. mRNA Expression Data for Markers of Myogenic Inhibition for the CHO, CHO+BCAA, CHO+LEU, and PLC Groups.

Variable	Pre	30 Min Post-Ex	120 Min Post-Ex	360 Min Post-Ex	G	T	G×T
Myostatin					**0.001 ***	0.38	0.13
CHO	1.04 ±0.11	1.00 ±0.10	0.98 ±0.09	0.99 ±0.07	**> CHO+LEU, PLC**		
CHO+BCAA	1.07 ±0.09	1.06 ±0.10	1.04 ±0.07	1.05 ±0.06	**> CHO+LEU, PLC**		
CHO+LEU	0.88 ±0.07	0.92 ±0.08	0.93 ±0.07	0.95 ±0.06			
PLC	0.84 ±0.06	0.89 ±0.05	0.95 ±0.06	0.98 ±0.04			
ACTRIIB					**0.001 ***	0.32	0.91
CHO	1.06 ± 0.06	1.06 ±0.09	1.08 ±0.04	1.03 ±0.03			
CHO+BCAA	0.97 ±0.05	0.96 ±0.04	1.01 ±0.05	0.98 ±0.04	**< CHO**		
CHO+LEU	0.91 ±0.03	0.98 ±0.06	0.98 ±0.04	0.96 ±0.07	**< CHO**		
PLC	1.01 ±0.06	1.03 ±0.04	1.02 ±0.05	1.00 ±0.03	**< CHO**		
P21cip		**> PRE**	**> PRE**	**> PRE**	0.27	**0.009 †**	0.47
CHO	1.03 ±0.03	1.05 ±0.06	1.08 ±0.10	1.08 ±0.11			
CHO+BCAA	1.03 ±0.10	1.05 ±0.09	1.04 ±0.08	1.04 ±0.05			
CHO+LEU	0.94 ±0.03	1.10 ±0.10	1.12 ±0.09	1.11 ±0.09			
PLC	1.04 ±0.03	1.10 ±0.09	1.10 ±0.07	1.11 ±0.06			
P27kip					0.37	0.37	0.73
CHO	1.06 ±0.12	1.05 ±0.11	1.03 ±0.10	1.02 ±0.07			
CHO+BCAA	1.08 ±0.09	1.03 ±0.08	0.99 ±0.08	1.01 ±0.07			
CHO+LEU	1.02 ±0.03	1.09 ±0.11	1.08 ±0.06	1.03 ±0.05			
PLC	1.13 ±0.10	1.05 ±0.09	1.07 ±0.03	1.04 ±0.05			

Data are presented as means ±SD for the ratio of Ct values for β-actin and each target mRNA variable. G, T, and G×T represent the univariate main effects for group, time, and group × time interaction, respectively, at a probability level of ≤ 0.05. The symbol * denotes a significant main effect for group. For myostatin, CHO and CHO+BCAA were significant greater than CHO+LEU and PLC. For ActRIIB, CHO was significantly greater than CHO+BCAA, CHO+LEU, and PLC, while CHO+LEU was greater than PLC. The symbol † denotes a significant main effect for time. PRE was significantly less than 30, 120, and 360 min post-exercise.

mRNA Expression for Markers of Myogenic Inhibition

Upon further analysis with univariate ANOVA, signifcant group main effects were observed for myostatin ($p = 0.001$; effect size = 1.00) and ActIIB ($p = 0.001$; effect size = 0.99). Post-hoc analysis showed that for myostatin CHO and CHO+BCAA were significantly greater than CHO+LEU and PLC. For ActIIB, CHO was significantly greater than CHO+BCAA, CHO+LEU, and PLC.

Analysis of the time main effects also produced significant differences between time points for p21cip ($p = 0.009$; effect size = 0.829). Post-hoc analysis showed that 30, 120, and 360 min post-exercise were significantly greater than PRE (Table 5).

DISCUSSION

The primary purpose of this study was to determine if the co-ingestion of carbohydrate with either BCAA or L-leucine in conjunction with a single bout of resistance exercise had any preferential effect to placebo or carbohydrate or placebo on serum IGF-1 and skeletal muscle mRNA expression of markers involved in activiating and inhibiting myogenic activation of satellite cells. In general, we observed resistance exercise to have no effect on serum IGF-1, but there was an effect on the expression of several genes. However, we observed no preferential RE-induced effects from the ingestion of any of the supplements for the variables assessed.

Since IGF-1 is known to be a humoral myogenic activator based on its binding and subsequent activation of its respective transmembrane receptor, we were compelled to determine the responsiveness of serum IGF-1 to resistance exercise and/or CHO and amino acid ingestion. In the present study, the response in the supplement groups for serum IGF-1 was not related to RE. This is congruent with our previous work using the same resistance exercise protocol, which also revealed no exercise-induced effects on the hepatically-derived release of IGF-1 (Taylor et al.

2012). An earlier study also demonstrated the unresponsiveness of serum IGF-1 to a single bout of resistance exercise (Kraemer et al. 1992).

We also suspected that insulin may also play a role in myogenically-related gene expression by virtue of the role that insulin receptor substrate 1 (IRS-1) autophosphorylation plays in increasing ERK 1/2 and Akt-mTOR signaling relative to transcriptional and translational mechanisms. Although not shown in the present study, our previous study (Ferreira et al. 2014) demonstrated significant increases in serum insulin compared to PLC that peaked at the 30 min post-exercise sampling point for both CHO-containing supplement groups. We observed the same response for the CHO+LEU group in the present study. Furthermore, none of these three intervention groups differed in regards to insulin levels (data not shown). Therefore, there were no preferential interactive effects from exercise and nutritional intervention on serum insulin or IGF-1. As a result, since there were no RE-related effects in any of the supplement groups on the expression of any of our targeted genes, the impact that either of these hormones played on the pre-translational activation of our selected genes appears to be negligible.

A single bout of heavy RE is sufficient to induce a transient up-regulation of muscle-specific gene expression (Wilborn et al. 2009; Willoughby & Nelson, 2002). Following a single bout of RE, the mRNA expression of the myogenic activators, Myo-D, myogenin, MRF-4, and myf5 were up-regulated at 30, 120, and 360 min post-exercise (Psilander et al. 2002; Wilborn et al. 2009; Willoughby & Nelson, 2002), and Cyclin B1 was significantly up-regulated 24 hr post- exercise (Kim et al. 2005; Wilborn et al. 2009). In older men, Myo-D, myogenin, and p27kip have been shown to be unresponsive to a single bout of RE when assessed one hr post-exercise (Hulmi et al. 2007). In agreement with previous results, in the present study we observed RE-induced increases in the mRNA expression of Cyclin B1 at 30 and 120 min post-exercise and Cyclin D1 at 120 and 360 min post-exercise. We also observed a strong trend towards significance in response to RE for MRF-4 based on p-value of 0.07 and a corresponding effect size of 0.58, which suggests this gene to be somewhat responsive to our RE intervention. Relatedly, in response to 45 min of treadmill running at 77% of VO2max, MRF-4 was increased in human vastus lateralis 4 hr post-exercise, while myogenin was unaltered (Harber et al. 2009)

A single bout of RE has also been shown to down-regulate the myogenic inhibitors, myostatin, and p27kip (Hulmi et al. 2008; Kim et al. 2005; Wilborn et al. 2009). Additionally, treadmill running has also been shown to result in a decrease in myostatin mRNA expression at 240 min post-exercise (Harber et al. 2009). However, in middle-aged men a single bout of RE has been shown to increase p21cip 60 min post-exercise (Hulmi et al. 2008). In older men, a single bout of RE exercise has been shown to down-regulate myostatin and ActIIB mRNA (Hulmi et al. 2007). Herein, we demonstrate a responsiveness to resistance exercise, as p21cip mRNA was up-regulated at 30, 120, and 360 min post-exercise.

Relative to the intake of nutrients, previous investigations have demonstrated conflicting results regarding the immediate ingestion of amino acids and/or carbohydrate after endurance- and resistance-related exercise. For example, the ingestion of 8.6 g of BCAAs (7 g leucine) one hr following RE resulted in a decreased myostatin mRNA expression at 180 and 360 min post-exercise. This occurred with concomitant increases in myogenin at 180 and 360 min post-exercise and Myo-D at 360 min post-exercise (Drummond et al. 2009). In middle-aged men, it was demonstrated that 15 g of whey isolate (~ 1.6 g of leucine) in conjunction with RE had no differential effect from placebo relative to the observed decreases in myostain mRNA expression at 60 min post-exercise (Hulmi et al. 2008). In addition, p21cip was increased at 60 min post-exercise compared to baseline for placebo but not the whey protein group. Following 60 min of cycle ergometry at 72% of VO2max during periods of either fasting or a meal containing 62 g of carbohydrate and approximately 9 g leucine, 5 g isoleucine, and 6 g valine, MRF-4 mRNA expression was increased at 120 and 360 min post-exercise during the fasting condition only (Har-

ber et al. 2010). In a study involving the ingestion of 10 g EAA (BCAA content equaling 3.5 g leucine, 0.8 g isoleucine, 1.0 g valine), but with no exercise intervention, myostatin mRNA was decreased and Myo-D increased 180 min post-ingestion (Drummond et al. 2009).

In the present study, we have attempted to delineate a possible preferential role that leucine might play compared to carbohydrate and/or BCAA in regards to the expression of genes involved in myogenic activity of satellite cells. Our present results, along with a number of previous studies, have provided compelling evidence that various genes serving as markers of the myogenic activity of satellite cells are load-sensitive, irrespective of muscle contractions involved in either resistance or endurance exercise. However, in line with previous studies our observation substantiate the lack of congruence that exists regarding the effectiveness of amino acid ingestion on pre-translational markers indicative of the myogenic activity of satellite cells.

In the present study, the three experimental groups received the equivalent amount of carbohydrate. The amino acid-containing groups both received the equivalent amount of leucine, which was provided at a relative dose of 60 mg/kg, and based on the range in body mass (approximately 67-95 kg), constituted an approximate leucine dose in the range of 4-6 g. This is unlike the previously-mentioned studies which provided absolute doses of amino acids and/or leucine ranging anywhere between 2-9 g. Based on the differences in macronutrient intakes, particularly amino acids, attempting to confidently corroborate our results with previous studies is challenging. Relative to the intake of amino acids, with the exception of cyclin D1, we have shown no preferential RE-induced effect from carbohydrate, BCAA, or L-leucine intake on serum IGF-1 or any of the gene targeted for analysis that are known markers in either the activation or inhibition of satellite cell activity. In regard to cyclin D1, in the absence of any carbohydrate and/or amino acid-induced effect on any of the other genes assessed, it is difficult to interpret the importance of this result.

Even though the role of carbohydrate and/or amino acids has not been conclusively demonstrated, the present study helps to solidify the understanding regarding the effectiveness of resistance exercise in regulating the pre-translational activity of markers of satellite cell activity. Therefore, in light of the results presented herein, we conclude that the co-ingestion of carbohydrate with either BCAA or L-leucine in conjunction with RE has no preferential effect on serum IGF-1 or pre-translational markers indicative of myogenesis.

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