The Journal of Nutrition
Nutrient Physiology, Metabolism, and Nutrient-Nutrient Interactions

The Leucine Content of a Complete Meal Directs Peak Activation but Not Duration of Skeletal Muscle Protein Synthesis and Mammalian Target of Rapamycin Signaling in Rats[1,2]

Layne E. Norton,[3]* Donald K. Layman,[3] Piyawan Bunpo,[5] Tracy G. Anthony,[5] Diego V. Brana,[4] and Peter J. Garlick[3,4]

[3]Division of Nutritional Sciences, Department of Food Science and Human Nutrition, and [4]Department of Animal Sciences University of Illinois at Urbana-Champaign, Urbana, IL 61801; and [5]Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Evansville, IN 47712

Abstract

This study examined the impact of leucine (Leu) derived from complete meals on stimulation of skeletal muscle protein synthesis (MPS). Expt. 1 examined time course changes in translation initiation and MPS after a meal. Male rats (~300 g) were trained for 5 d to eat 3 meals/d providing 20, 50, and 30% of energy from whey protein, carbohydrates, and fats, respectively. Plasma and skeletal muscle were collected at time 0 (baseline) after 12 h of food deprivation and then at 45, 90, 135, 180, and 300 min after a 4-g meal. Plasma Leu increased at 45 min and remained elevated through 180 min. MPS peaked at 45–90 min and returned to baseline by 180 min. Plasma Leu correlated with phosphorylation of ribosomal protein p70 S6 kinase ($r = 0.723$; $P < 0.05$), eukaryotic initiation factor 4E binding protein-1 ($r = 0.773$; $P < 0.05$), and MPS ($r = 0.608$; $P < 0.05$) over time. Expt. 2 examined 3 levels of protein intake (10, 20, and 30% of energy) from 2 sources (wheat and whey) with different Leu contents (~6.8 and ~10.9%, respectively) on stimulation of initiation and MPS. Rats were trained to eat 3 meals/d providing 14, 56, and 30% of energy from protein, carbohydrates, and fats. On d 6, MPS was evaluated at 90 min after rats consumed 1 of the 6 test meals. Whey protein stimulated initiation and MPS more than wheat and the differential response related to greater plasma Leu responses in the whey groups. These studies demonstrate that peak activation but not duration of MPS is proportional to the Leu content of a meal. J. Nutr. 139: 1103–1109, 2009.

Introduction

Leucine (Leu) is a unique regulator of muscle protein synthesis (MPS)[6] and translation initiation and has been proposed as a key mechanism translating diet quality into the meal response of MPS (1,2). Leu alone can stimulate protein synthesis to the same extent as a complete protein or complete mixture of amino acids by activating several factors involved with initiating mRNA translation, primarily through the mammalian target of rapamycin (mTOR) signaling pathway, including ribosomal protein S6 kinase (S6K), and eukaryotic initiation factor (eIF) 4E binding protein-1 (4E-BP1) in a dose-dependant fashion (3,4).

While Leu's stimulatory effects on MPS and translation initiation have been extensively documented in vivo using purified amino acids, the impact of Leu when consumed as part of intact protein in complete meals is unknown. Further, neither the relationship of Leu in complete meals with the postprandial time course of MPS nor the impact of meals containing isonitrogenous protein sources with different Leu contents on MPS has been investigated.

The time course of MPS in response to an oral dose of free Leu has been previously characterized by Anthony et al. (4) with synthesis peaking from 30 to 60 min after Leu administration and returning to control levels by 120 min. The ability of Leu to stimulate MPS suggests that Leu content may be an important criterion for defining optimum protein quantity and quality for a meal. Low-protein meals (~10 g/meal) with limited Leu content produce minimal increases in plasma Leu in humans compared with higher protein meals (~30 g/meal) (5). It is not well understood whether feeding different Leu amounts as part of isonitrogenous complete meals produces differences in plasma Leu, translation initiation, mTOR signaling, and MPS.

The goal of this study was 2-fold: first, to define the time course of plasma and intracellular Leu concentrations following consumption of a complete meal containing a Leu-rich protein source and to correlate these responses with mTOR signaling,

[1] Supported by grants from the National Dairy Council/DMI, Illinois Department of Agriculture (CFAR), and Egg Nutrition Center.
[2] Author disclosures: L. E. Norton, D. K. Layman, P. Bunpo, T. G. Anthony, D. V. Brana, and P. J. Garlick, no conflicts of interest.
[6] Abbreviations used: 4E-BP1, eukaryotic initiation factor 4E binding protein-1; EAA, essential amino acids; eIF, eukaryotic initiation factor; FSR, factional rate of protein synthesis; MPS, skeletal muscle protein synthesis; mTOR, mammalian target of rapamycin; S6K, ribosomal protein p70 S6 kinase.
* To whom correspondence should be addressed. E-mail: lnorton2@illinois.edu.

0022-3166/08 $8.00
Manuscript received December 22, 2008. Initial review completed January 17, 2009. Revision accepted March 24, 2009.
First published online April 29, 2009; doi:10.3945/jn.108.103853.

translation initiation factors, and MPS; and second, to assess how meals containing 3 different levels of wheat or whey proteins (10, 20, and 30% of total energy) with different Leu contents (6.8 and 10.9% Leu, respectively) affect plasma amino acid profiles, mTOR signaling, and MPS.

Materials and Methods

Animals and diets. The animal facilities and protocol were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Illinois at Urbana-Champaign.

Expt. 1 examined the time course of changes in plasma Leu, MPS, and translation initiation factors after a meal. Male Sprague-Dawley rats (300 ± 15 g; Harlan-Teklad) were maintained at 24°C with a 12-h-light:-dark cycle and free access to water. Rats were fed diets providing 20, 50, and 30% of energy from whey protein, carbohydrates, and fats, respectively (**Table 1**).

Rats were trained to meal feed using 3 meals/d consisting of a small (4 g) meal consumed between 0700 and 0720 followed by ad libitum consumption of diets from 1300 to 1400 and 1800 to 1900 (5). Rats consumed ~19 g/d of total diet. After 5 d of meal training, rats were food deprived for 12 h (from 1900 to 0700) and then examined at 6 time points: before the first meal (time 0) and at 45, 90, 135, 180, and 300 min after the 4-g meal. At the indicated times, rats were killed and blood and tissue samples collected. Gastrocnemius, soleus, and plantaris muscles were excised and immediately frozen in liquid nitrogen for later analysis.

Expt. 2 compared the potential of wheat protein and whey protein to stimulate MPS and translation initiation in skeletal muscle at 3 levels of protein intake (10, 20, and 30% of energy from protein) at 90 min after the test meal, representing the peak level of MPS in Expt. 1. The 2 protein sources were selected to provide the same amount of total nitrogen with different levels of Leu. Leu accounts for ~10.9% of the total amino acids in whey protein and 6.8% in wheat gluten (**Table 2**). Male rats (250 ± 12 g) were maintained similar to Expt. 1 except they were adapted to meal feeding using a control diet providing 14, 56, and 30% of energy from whey protein, carbohydrates, and fats, respectively (Table 1). After 5 d of meal feeding, rats were randomized into either control ($n = 7$) or 1 of the 6 treatment groups: 10% wheat protein ($n = 8$), 10% whey protein ($n = 8$), 20% wheat protein ($n = 8$), 20% whey protein ($n = 8$), 30% wheat protein ($n = 8$), and 30% whey protein ($n = 8$) (Tables 1 and 2). Wheat gluten diets were supplemented with lysine to meet NRI requirements and to approximate whey lysine levels (Table 1) (6). Rats were food deprived for 12 h and then provided a single 4-g test meal from 1 of the 6 experimental diets. The test meals contained ~0, 29, 47, 60, 94, 89, and 142 mg of Leu, respectively, for the control, 10% wheat, 10% whey, 20% wheat, 20% whey, 30% wheat, and 30% whey groups. Rats were killed 90 min after consumption of the meal and blood and tissue samples were collected.

TABLE 1 Composition of test diets

		10%		20%		30%	
Component	Baseline diet[1]	Wheat	Whey	Wheat	Whey[2]	Wheat	Whey
				g/kg			
Vital wheat gluten[3]	0.0	132.0	0.0	256.0	0.0	378.0	0.0
L-Lysine supplement[4]	0.0	7.2	0.0	14.4	0.0	21.6	0.0
Whey protein isolate[5]	159.6	0.0	115.2	0.0	228.0	0.0	340.6
Cornstarch	357.4	377.8	401.8	246.6	290.0	117.4	176.4
Maltodextrin	134.1	134.1	134.1	134.1	134.1	134.1	134.1
Sucrose	101.5	101.5	101.5	101.5	101.5	101.5	101.5
Soybean oil	140.9	140.9	140.9	140.9	140.9	140.9	140.9
Cellulose (fiber)	53.7	53.7	53.7	53.7	53.7	53.7	53.7
Mineral mix[6]	37.6	37.6	37.6	37.6	37.6	37.6	37.6
Vitamin mix[6]	10.7	10.7	10.7	10.7	10.7	10.7	10.7
Choline bitautrate	2.7	2.7	2.7	2.7	2.7	2.7	2.7

[1] Diet used to adapt rats to meal feeding in Expt. 2.
[2] Diet used in Expt. 1.
[3] Vital wheat gluten purchased from Honeyville Grain, Honeyville, UT. 75.5% protein, 15.5% carbohydrate, 9% other.
[4] Vital wheat gluten supplemented with 6.3 g L-lysine/100 g protein to match whey protein isolate.
[5] Whey protein provided by Perham, Perham, MN. 92.1% protein, 1.6% carbohydrate, 6.3% other.
[6] Mineral and vitamin supplements (25) from Harlen-Teklad, Madison, WI.

TABLE 2 Amino acid compositions of protein sources

Amino acid	Wheat[1]	Whey[2]
	g/100 g protein	
Alanine	3.1	4.9
Arginine	4.7	2.4
Aspartate	4.0	10.6
Cysteine	1.9	2.5
Glutamate/glutamine	31.7	16.9
Glycine	3.8	1.8
Histidine	1.8	2.0
Isoleucine	3.0	6.2
Leucine	6.8	10.9
Lysine[3]	2.8 (+6.3)	9.1
Methionine	1.9	2.0
Phenylalanine	4.4	3.3
Proline	9.4	5.6
Serine	3.9	4.7
Threonine	2.6	6.4
Tryptophan	1.3	1.7
Tyrosine	2.4	3.0
Valine	4.5	6.0

[1] Vital wheat gluten purchased from Harlan Teklad, Madison, WI. 76% protein, 15.2% carbohydrate, 8.8% other.
[2] Whey protein isolate provided by Perham, Perham, MN. 92.1% protein, 1.6% carbohydrate, 6.3% other.
[3] Wheat gluten supplemented with 6.3 g L-lysine/100 g protein to match whey protein.

Determination of MPS. Protein synthesis was measured in skeletal muscle using the flooding dose method (7). A 40% enriched L-[$^2H_5$]phenylalanine solution (150 mmol/L; Cambridge Isotopes) was administered at 150 μmol/100 g body weight and injected via the tail vein (1 mL/100g body weight). After 10 min, animals were killed by decapitation and hind limbs quickly removed and immersed in an ice-water mixture. Gastrocnemius, plantaris, and soleus muscles were removed from cooled hind limbs, frozen in liquid N_2, and stored at −80°C.

Frozen muscle tissue was powdered in liquid nitrogen and protein was precipitated with cold (4°C) perchloric acid (30 g/L, 1 mL/50 mg muscle tissue). The resulting supernatant and protein pellet were prepared for analysis by GC-MS as described previously (8,9). The enrichment of L-[$^2H_5$]phenylalanine in the muscle hydrolysate was measured by GC-MS using a 6890N GC and a 5973N mass detector (Agilent Technologies). The samples were analyzed under electron impact ionization and in splitless mode, and phenylethylamine ions at mass:charge ratio 106 (m + 2) and 109 (m + 5) were monitored for enrichment analysis.

The muscle supernatant was used to determine intracellular free phenylalanine enrichment. Free amino acids were purified by ion exchange resin solid-phase extraction using EZ:faast amino acid analysis sample testing kit (Phenomenex) and $^2H_5$-phenylalanine enrichment was determined using a propyl chloroformate derivative with GC-MS by monitoring the ions at mass:charge ratio 206 (m) and 211 (m + 5) (10).

We assessed fractional rates of protein synthesis (FSR) from the rate of incorporation of L-[$^2H_5$]phenylalanine into total mixed muscle protein as described previously (8). The time from injection of the metabolic tracer until tissue cooling was recorded as the actual time for L-[$^2H_5$]phenylalanine incorporation. FSR, defined as the percentage of tissue protein renewed each day, were calculated according to the formula: FSR = $(E_b \times 100)/(E_a \times t)$, where t is the time interval between injection and cooling of sampled tissue expressed in days and E_b and E_a are the enrichments of [$^2H_5$]Phe in hydrolyzed tissue protein and in muscle free amino acids, respectively.

Plasma measurements. Plasma was obtained from trunk blood by centrifugation at 1800 × *g*; 10 min at 4°C. Plasma insulin concentrations were analyzed using a commercial RIA kit for rat insulin (Linco Research). Plasma amino acid concentrations were analyzed by HPLC using a Waters 2475 Fluorescence detector (11).

Quantitation of eIF4E, 4E-BP1·eIF4E, and eIF4G·eIF4E complexes. The association of 4E-BP1 and eIF4G with eIF4E in muscle extracts was examined by protein immunoblot analysis as previously described (6,12–14). Membranes were blotted using a rabbit polyclonal antibody to eIF4E (Santa Cruz Biotechnology), eIF4G (Bethyl Labs), or 4E-BP1 (Bethyl Labs).

Phosphorylation of 4E-BP1 and S6K. Muscle supernatants were subjected to protein immunoblot analysis as described previously (6, 12–14) using a rabbit polyclonal antibody to 4E-BP1 (Bethyl Labs) and a rabbit polyclonal S6K antibody (Bethyl Labs).

Statistical analysis. All data were analyzed by SPSS 15.0 software package for Windows. We performed a 1-way ANOVA with the postprandial time as the independent variable for Expt. 1. A 2-way ANOVA was performed with treatment group as the independent variable for Expt. 2. When a significant overall effect was detected, differences among individual means were assessed using Fisher's least significant difference post hoc test. Separate comparisons to baseline were made using a *t* test. All data sets were tested for normal distribution and variance homogeneity using Levene's test. When variances were not homogeneous, means were compared using a Games-Howell test. Correlation between data were determined by linear regression (Pearson correlation). The level of significance was set at $P < 0.05$ for all statistical tests. Values in the text are means ± SEM.

Results

Expt. 1. Time course changes in MPS, plasma Leu, and the activity of protein factors involved in regulating mRNA translation were examined at 0, 45, 90, 135, 180, and 300 min in rats after consuming a 4-g complete meal containing 20% whey protein (providing ~94 mg of Leu). MPS increased from 0 to 45 min after the meal and reached peak values at 45 and 90 min postprandial (**Fig. 1**). From 90 to 135 min, MPS values decreased significantly and returned to baseline values at 180 min.

Plasma Leu increased at 45 min after the meal and remained elevated through 300 min, with peak values from 45 to 180 min (**Fig. 2***A*). Intracellular Leu concentrations changed in a pattern similar to plasma Leu (Fig. 2*B*). Plasma isoleucine, valine, lysine, and methionine followed similar postprandial patterns and concentrations returned to baseline by 300 min (**Table 3**).

Plasma insulin concentrations increased significantly at 90 min (Table 3) then returned to baseline by 180 min. Plasma glucose did not differ from baseline at any point during the time course (data not shown).

Time course changes in 4E-BP1 and S6K were measured to evaluate the mTOR signaling pathway. Both 4E-BP1 (**Fig. 3***A*) and S6K (Fig. 3*B*) phosphorylation increased between 45 min and 180 min. At 300 min, both 4E-BP1 and S6K phosphorylation declined from peak activation; 4E-BP1 did not differ from baseline, whereas S6K phosphorylation remained at ~50% of peak activation.

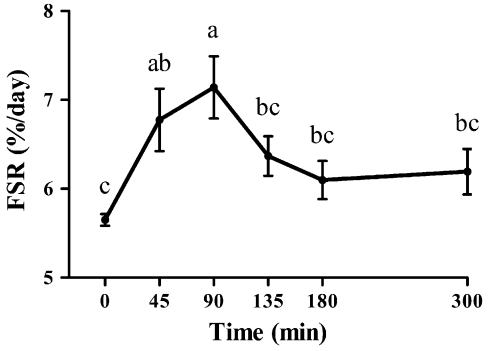

FIGURE 1 Time course changes in the FSR in gastrocnemius muscle of rats fed a 4-g complete meal containing 20, 50, and 30% of energy from protein, carbohydrates, and fats, respectively. Data are means ± SEM; $n = 5$–6. Labeled means without a common letter differ, $P < 0.05$.

We evaluated the formation of the eIF4F translation initiation complex by determining the association of eIF4E with eIF4G. Association of eIF4E with eIF4G increased from 90 to 135 min ($P < 0.05$), tended to be greater at 180 min ($P = 0.055$), and then had returned to baseline at 300 min (Fig. 3*C*).

Changes in MPS were correlated with plasma and intracellular Leu levels during the early postprandial period. From 0 to 90 min after the meal, plasma Leu correlated ($P < 0.001$) with activation of S6K ($r = 0.858$), 4E-BP1 ($r = 0.802$), and MPS ($r = 0.782$). The correlation of plasma Leu with MPS was less strong from 90 to 300 min postprandial ($r = 0.492$; $P < 0.01$). MPS had a weak correlation with plasma insulin over time ($r = 0.424$; $P < 0.01$).

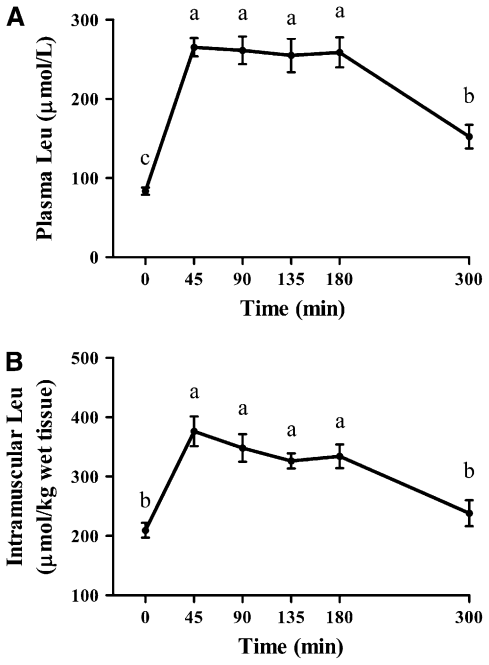

FIGURE 2 Time course changes in plasma (*A*) and intramuscular (*B*) Leu concentrations of rats fed a 4-g complete meal containing 20, 50, and 30% of energy from protein, carbohydrates, and fats, respectively. Data are means ± SEM; $n = 5$–6. Labeled means without a common letter differ, $P < 0.05$.

TABLE 3 Time course changes in selected EAA and insulin concentrations in rats fed a complete meal[1]

	Time, *min*					
	0[2]	45	90	135	180	300
			μmol/L			
Leucine	83.6 ± 4.6^{c}	265 ± 11.6^{a}	261 ± 17.4^{a}	255 ± 21.3 ^{a}	258 ± 19.0^{a}	152 ± 15.1^{b}
Isoleucine	55.6 ± 2.8^{b}	121 ± 5.5^{a}	111 ± 8.9^{a}	108 ± 14.9^{ab}	108 ± 9.8^{a}	93.2 ± 6.6^{ab}
Valine	143 ± 8.9^{b}	227 ± 5.5^{a}	223 ± 13.4^{a}	227 ± 13.5^{a}	235 ± 14.6^{a}	159 ± 13.4^{b}
∑ BCAA	282 ± 16.0^{b}	615 ± 21.3^{a}	595 ± 39.0^{ab}	591 ± 67.6^{ab}	602 ± 42.9^{ab}	372 ± 43.6^{b}
Lysine	472 ± 26.8^{c}	1074 ± 176.9^{bc}	855 ± 91.6^{ab}	852 ± 50.7^{ab}	647 ± 38.0^{ab}	385 ± 7.3^{c}
Methionine	27.0 ± 1.1^{c}	52.3 ± 3.0^{a}	43.9 ± 3.1^{a}	41.7 ± 4.6^{ab}	34.6 ± 2.8^{bc}	30.7 ± 2.1^{bc}
Threonine	556 ± 61.0^{b}	840 ± 65.9^{a}	843 ± 43.2^{a}	921 ± 57.8^{a}	917 ± 64.2^{a}	785 ± 48.3^{a}
Insulin, *pmol/L*	77.3 ± 14.9^{b}	89.0 ± 15.7^{b}	156 ± 36.1^{a}	137 ± 17.3^{ab}	90.2 ± 9.2^{b}	96.2 ± 6.6^{b}

[1] Values are means ± SEM, $n = 5$–6. Labeled means without a common letter differ, $P < 0.05$.
[2] 12-h food-deprived controls.

Expt. 2. Expt. 2 compared the potential of wheat protein and whey protein at 3 dietary levels of protein intake (10, 20, and 30% of energy from protein) to stimulate MPS and translation initiation in skeletal muscle at 90 min representing the peak level of postprandial MPS in Expt. 1. Plasma Leu increased significantly above food-deprived controls in 10% whey but not 10% wheat (**Fig. 4**). Within the 10, 20, and 30% protein treatment groups, whey protein produced higher plasma Leu concentrations than wheat, except for the 30% group. Plasma concentrations of isoleucine and valine were also greater in the whey groups compared with the wheat groups, except at the 30% protein level (**Table 4**). Plasma concentrations of lysine, methionine, and threonine did not differ between wheat and whey for any of the groups examined with the exception of methionine, which was significantly lower in the 10% wheat group than in the 10% whey group (Table 4). Plasma lysine and threonine increased above baseline at the 20 and 30% protein levels ($P < 0.05$) with no significant difference between whey and wheat.

Phosphorylation of 4E-BP1 (**Fig. 5***A*) and S6K (Fig. 5*B*) increased after all test meals. However, whey protein promoted mTOR signaling better than wheat protein at nearly all protein levels.

MPS increased after all meals except the 10% wheat group (**Fig. 6**). Consistent with mTOR signaling targets 4E-BP1 and S6K, the main effect was whey protein increased MPS more than wheat protein at equal protein intakes, with the greatest individual differences in the 10% ($P < 0.05$) and 30% ($P = 0.062$) protein groups, where whey stimulated MPS 52 and 19% more than wheat, respectively.

Plasma insulin concentrations increased after the meals except for the 10% whey and 20% wheat groups ($P < 0.05$).

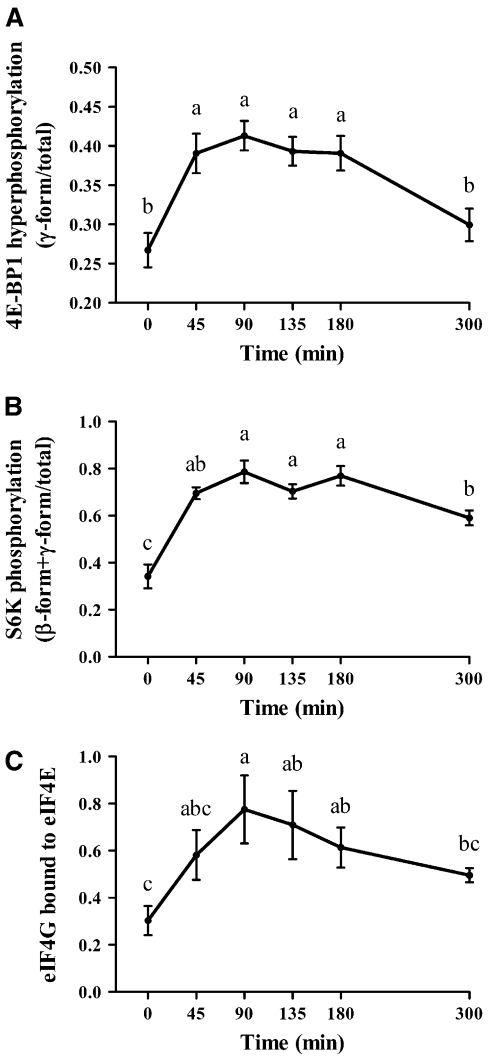

FIGURE 3 Time course changes in the phosphorylation state of 4E-BP1 (*A*), p70S6K (*B*), and binding of eIF4G to eIF4E (*C*) in gastrocnemius muscle of rats fed a 4-g complete meal containing 20, 50, and 30% of energy from protein, carbohydrates, and fats, respectively. Data are means ± SEM; $n = 5$–6. Labeled means without a common letter differ, $P < 0.05$.

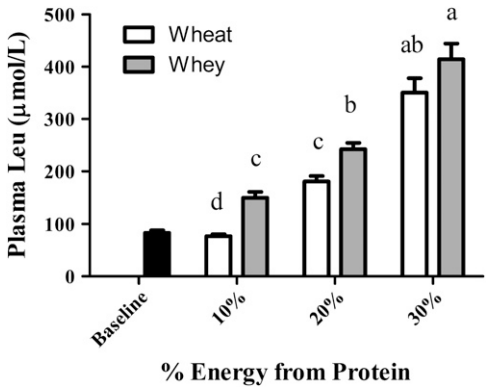

FIGURE 4 Plasma Leu concentrations of rats fed complete meals containing either wheat or whey at 3 different total protein contents (10, 20, or 30% of energy). Data are means ± SEM; $n = 7$–8. Labeled means without a common letter differ, $P < 0.05$. All fed groups except 10% wheat differed from baseline, $P < 0.05$.

TABLE 4 Selected plasma EAA and insulin concentrations in rats 90 min after feeding complete meals containing wheat or whey[1]

		10%		20%		30%	
	Baseline[2]	Wheat	Whey	Wheat	Whey	Wheat	Whey
Leucine	83.1 ± 4.8	76.9 ± 3.9^{d}	149.5 ± 11.0^{c}	180.7 ± 10.3^{c}	242.3 ± 12.2^{b}	350.7 ± 27.3^{ab}	442.8 ± 12.2^{a}
Isoleucine	44.5 ± 2.3	50.6 ± 2.5^{d}	101.5 ± 9.3^{c}	121.5 ± 9.8^{bc}	155.1 ± 12.7^{b}	223.7 ± 19.6^{ab}	257.5 ± 17.5^{a}
Valine	118.0 ± 4.6	83. 6 ± 4.4^{c}	190.5 ± 18.2^{b}	211.2 ± 13.4^{b}	319.8 ± 35.3^{ab}	430.7 ± 39.1^{a}	505.4 ± 40.1^{a}
∑ BCAA	245.6 ± 11.0	211.1 ± 10.4^{c}	441.5 ± 37.4^{b}	513.4 ± 32.2^{b}	717.3 ± 51.1^{ab}	967.8 ± 77.2^{a}	1177.2 ± 84.2^{a}
Lysine	449.3 ± 39.9	394.5 ± 26.1^{b}	530.1 ± 65.0^{ab}	663.7 ± 69.5^{ab}	821.8 ± 84.4^{a}	705.1 ± 70.1^{ab}	658.2 ± 49.8^{ab}
Methionine	44.1 ± 3.9	31.0 ± 0.9^{c}	46.8 ± 3.8^{ab}	60.2 ± 6.4^{ab}	59.8 ± 4.6^{ab}	70.9 ± 7.0^{a}	77.9 ± 8.8^{a}
Threonine	568.2 ± 53.1	536.2 ± 46.6^{c}	670.6 ± 109.2^{bc}	766.3 ± 74.2^{b}	902.4 ± 85.8^{ab}	1038.3 ± 88.3^{a}	1024.6 ± 71.4^{a}
Insulin *pmol/L*	76.4 ± 6.5	142.7 ± 15.9^{ab}	119.0 ± 24.3^{b}	118.1 ± 21.1^{b}	178.4 ± 20.2^{a}	171.7 ± 22.5^{ab}	134.6 ± 18.1^{ab}

[1] Values are means ± SEM, $n = 6$–8. Main effects for wheat vs. whey were significant. Labeled means without a common letter differ, $P < 0.05$.
[2] 12-h food-deprived control.

Peak values were obtained in the 20% whey and 30% wheat groups (Table 4).

Discussion

These experiments evaluated time course changes and peak activation in MPS, mTOR signaling, and changes in plasma amino acids in response to nutritionally complete meals with the purpose of determining the contribution of Leu to directing postprandial changes in MPS. The major conclusion of this work is that Leu is the primary factor directing postprandial mTOR signaling and peak stimulation of MPS, but the duration of the meal response in skeletal muscle is influenced by factors in addition to Leu concentrations.

Expt. 1 examined the time course of MPS after a meal containing 20% of energy from whey protein. MPS increased above baseline levels by 45 min with a peak value at 90 min and returned to baseline at 3 h postprandially. Previous research using purified amino acids demonstrated a more rapid MPS response that peaked at 45 min and returned to baseline by 2 h (3,4,15). Thus, feeding a complete meal appears to both delay and prolong MPS in skeletal muscle compared with oral intake of Leu or essential amino acids (EAA) (3,4).

These results provide support for the role of Leu as a key trigger for postprandial stimulation of MPS after a complete meal but indicate that elevated plasma Leu is not sufficient to produce sustained elevations of MPS. The cause of MPS becoming refractory to plasma Leu could not be explained by the mTOR targets 4E-BP1 and S6K, because they remained activated at 180 min postprandial. Likewise, the rate-limiting step of eIF4F assembly, eIF4E binding to eIF4G, is increased above baseline at 135 min, a point at which MPS is decreasing from peak activation.

The mechanism for the postprandial decline in MPS with elevated Leu is unknown (16). Layman and Wisont (17) demonstrated that Leu infusion in rats stimulated MPS but prolonged infusion-depleted plasma valine and isoleucine in

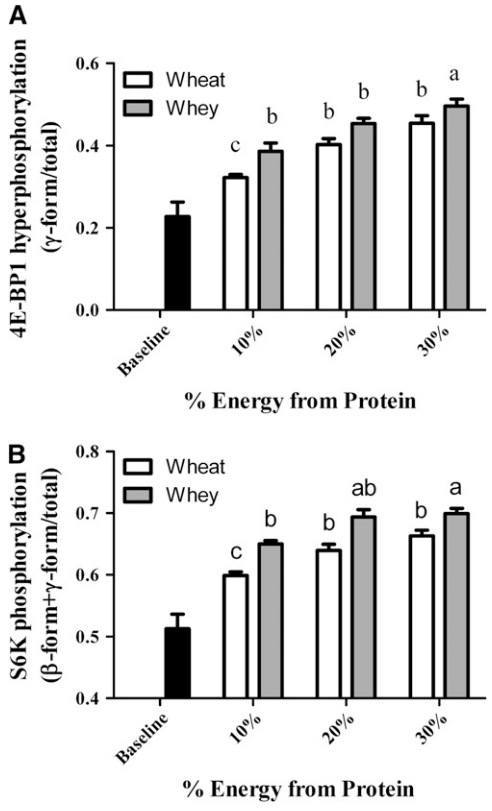

FIGURE 5 Phosphorylation states of 4E-BP1 (*A*) and p70S6K (*B*) in gastrocnemius muscle of rats fed a complete meal containing either wheat or whey at 3 different total protein contents (10, 20, or 30% of energy). Data are means ± SEM; $n = 7$–8. Labeled means without a common letter differ, $P < 0.05$. All fed groups differed from baseline, $P < 0.05$.

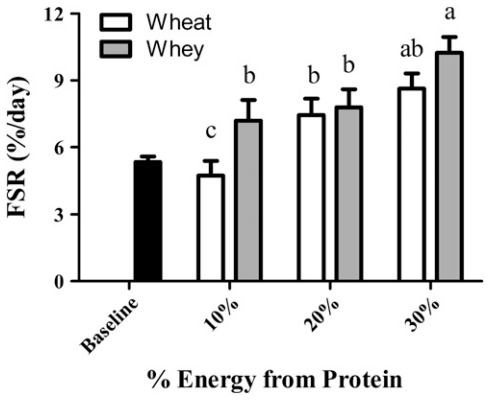

FIGURE 6 Rates of protein synthesis in gastrocnemius muscle of rats fed a complete meal containing either or whey at 3 different total protein contents (10, 20, or 30% of energy). Data are means ± SEM; $n = 7$–8. Labeled means without a common letter differ, $P < 0.05$. All fed groups except 10% wheat differed from baseline, $P < 0.05$.

proportion to the rate of substrate use for MPS. Coinfusion of valine and isoleucine prevented plasma depletion. Likewise, Escobar et al. (18) reported that Leu infusion in neonatal pigs depleted plasma EAA coincident with a decline in MPS. These investigators found that infusion of a complete mixture of EAA maintained MPS. Contrary to these findings, Bohe et al. (16) documented the refractory nature of skeletal muscle to elevations of amino acids during a 6-h infusion of a complete mixture of EAA. The infusion produced constant elevations in plasma EAA; however, the synthetic response lasted only 2 h and was unable to be further stimulated by infusion of additional EAA during the 6-h period. Likewise, in the current study, the refractory response to Leu occurred without a parallel decline in other EAA.

Another potential mechanism for the refractory response may include plasma insulin. Insulin is not required to stimulate protein synthesis in adults, but it does optimize the MPS response of muscle to amino acids (4,19). Bohe et al. (16) found that that plasma insulin time course somewhat paralleled the decline in MPS. Based on this, an elevation in insulin is perhaps necessary to sustain the anabolic response of MPS associated with elevated plasma Leu. If insulin contributes to the duration of MPS in the current study, the mechanism appears to be independent of insulin's effects on translation initiation, because phosphorylation of 4E-BP1 and S6K remained elevated at 180 min, whereas MPS and plasma insulin declined to baseline. The correlation of plasma insulin with MPS was significant over the time course but less than the correlation between Leu and MPS. Insulin also stimulates peptide elongation in skeletal muscle (20). Changes in plasma insulin concentrations may contribute to the refractory period of MPS through changes in peptide elongation. Taken together, current information is insufficient to explain why skeletal muscle becomes refractory to the anabolic effects of elevated amino acids.

Expt. 2 examined responses of translation initiation and MPS to isonitrogenous meals with different Leu contents. MPS and translation initiation are known to respond in a dose relationship to Leu administration (3,4), but to our knowledge, this was the first experiment to explore dosing Leu as a component of different protein sources in complete meals. Overall, whey protein with high Leu content was superior to wheat protein in initiating MPS in rats fed complete meals. These findings are consistent with a previous report showing that whey protein fed after exercise stimulated mTOR signaling to a greater extent than soy protein (12).

In accordance with the Leu contents of wheat and whey protein, plasma levels of Leu were greater at every level of protein intake for animals fed whey compared with wheat protein. Interestingly, feeding rats the 4-g meal containing 10% wheat protein (~29 mg Leu) did not increase plasma Leu levels. Consistent with the plasma data, 10% wheat did not stimulate MPS. Conversely, feeding a meal containing 10% whey (~47 mg Leu) almost doubled plasma Leu levels and stimulated MPS above baseline, suggesting a minimum threshold of Leu intake to increase plasma Leu concentration and initiate MPS after 90 min. Further, at 10% protein intake, 4E-BP1 and S6K were phosphorylated to a greater extent by whey protein than wheat protein. Interestingly, 10% wheat protein stimulated phosphorylation of 4E-BP1 and S6K above baseline values despite the lack of increase of plasma Leu. This may be attributed to the rise in plasma insulin in the 10% wheat group, which is also known to activate the mTOR pathway but to a reduced level in the absence of Leu (19).

A second factor that may contribute to differences in the postprandial rise in plasma amino acids is the rate of gastric emptying and peptide digestion and absorption (21). Whey proteins, which are highly soluble, empty rapidly from the stomach compared with casein (21); thus, it is possible that whey and wheat proteins may empty at different rates, which could have affected postprandial plasma Leu, MPS, and mTOR signaling responses. However, research from Bos et al. (21) demonstrated that plasma amino acids peak at ~60 min postprandially in humans, which is similar to the reported amino acid peak for whey protein in humans (22). This is consistent with the postprandial plasma Leu concentrations that are in proportion to the actual Leu content of each protein.

The time course of stimulation of MPS after a meal and the refractory nature of the anabolic response at 3 h provokes the question as to whether a second stimulation can be achieved with a new meal. Along these lines, these data suggest that the meal distribution of protein throughout the day is an important factor to consider in future experiments. Current dietary guidelines for protein do not mention distribution of intake throughout the day. Data from de Castro (23) indicate that Americans consume over 65% of their daily protein after 1830 h. It is unlikely that they reach a threshold of protein intake necessary to stimulate MPS at meals before 1830. Research from Arnal et al. (24) also supports the importance of a protein threshold at meals. Furthermore, if the anabolic response to a high-protein meal lasts only 3 h, then it is likely that a large portion of the day is spent in a catabolic state. This suggests that optimum protein intakes for adults require proteins rich in Leu to be provided in multiple meals throughout the day.

In summary, these data suggest that the Leu content of various protein sources is an important indicator of protein quality as it relates to acute stimulation of MPS. These data also provide further evidence that a specific threshold of Leu intake is needed to initiate mRNA translation and MPS and that low intake of some protein sources may not reach this threshold. Additionally, these data lead us to propose that the distribution of protein and specifically Leu intake throughout the day is an important factor to optimize the muscle anabolic response to each meal.

Acknowledgment

We thank Indu Rupassara for assistance with GC-MS analyses.

Literature Cited

1. Layman DK. The role of leucine in weight loss diets and glucose homeostasis. J Nutr. 2003;133:S261–7.
2. Norton LE, Layman DK. Leucine regulates translation initiation of protein synthesis in skeletal muscle after exercise. J Nutr. 2006;136: S533–7.
3. Crozier SJ, Kimball SR, Emmert SW, Anthony JC, Jefferson LS. Oral leucine administration stimulates protein synthesis in rat skeletal muscle. J Nutr. 2005;135:376–82.
4. Anthony JC, Lang CH, Crozier SJ, Anthony TG, MacLean DA, Kimball SR, Jefferson LS. Contribution of insulin to the translational control of protein synthesis in skeletal muscle by leucine. Am J Physiol Endocrinol Metab. 2002;282:E1092–101.
5. Layman DK, Shiue H, Sather C, Erickson DJ, Baum J. Increased dietary protein modifies glucose and insulin homeostasis in adult women during weight loss. J Nutr. 2003;133:405–10.
6. National Research Council. Nutrient requirements of the laboratory rat. In: Nutrient requirements of laboratory animals. National Academy of Sciences, Washington (DC): 1995. p 24–6
7. Garlick PJ, McNurlan MA, Preedy VR. A rapid and convenient technique for measuring the rate of protein-synthesis in tissues by injection of [^{3}H]phenylalanine. Biochem J. 1980;192:719–23.
8. McNurlan MA, Essén P, Thorell A, Calder AG, Anderson SE, Ljungqvist O, Sandgren A, Grant I, Ballmer PE, et al. Response of

protein synthesis in human skeletal muscle to insulin: an investigation with [$^2H_5$]phenylalanine. Am J Physiol. 1994;267:E102–8.
9. Essén P, McNurlan MA, Gamrin L, Hunter K, Calder G, Garlick PJ, Wernerman J. Tissue protein synthesis rates in critically ill patients. Crit Care Med. 1998;26:92–100.
10. Mohabbat T, Drew B. Simultaneous determination of 33 amino acids and dipeptides in spent cell culture media by gas chromatography-flame ionization detection following liquid and solid phase extraction. J Chromatogr B Analyt Technol Biomed Life Sci. 2008;862:86–92.
11. Reverter M, Lundh T, Lindberg JE. Determination of free amino acids in pig plasma by precolumn derivatization with 6-N-aminoquinolyl-N-hydroxysuccinimidyl carbamate and high-performance liquid chromatography. J Chromatogr B Biomed Sci Appl. 1997;696:1–8.
12. Anthony TG, McDaniel BJ, Knoll P, Bunpo P, Paul GL. Feeding meals containing soy or whey protein after exercise stimulates protein synthesis and translation initiation in the skeletal muscle of male rats. J Nutr. 2007;137:357–62.
13. Anthony JC, Anthony TG, Kimball SR, Vary TC, Jefferson LS. Orally administered leucine stimulates protein synthesis in skeletal muscle of postabsorptive rats in association with increased eIF4F formation. J Nutr. 2000;130:139–45.
14. Anthony TG, Anthony JC, Yoshizawa F, Kimball SR, Jefferson LS. Oral administration of leucine stimulates ribosomal protein mRNA translation but not global rates of protein synthesis in the liver of rats. J Nutr. 2001;131:1171–6.
15. Kimball SR, Jefferson LS. Molecular mechanisms through which amino acids mediate signaling through the mammalian target of rapamycin. Curr Opin Clin Nutr Metab Care. 2004;7:39–44.
16. Bohé J, Low JF, Wolfe RR, Rennie MJ. Latency and duration of stimulation of human muscle protein synthesis during continuous infusion of amino acids. J Physiol. 2001;15:575–9.
17. Layman DK, Wisont G. Leucine stimulates clearance of indispensable amino acids. FASEB J. 1999;13:A908.
18. Escobar J, Frank JW, Suryawan A, Nguyen HV, Davis TA. Amino acid availability and age affect the leucine stimulation of protein synthesis and eIF4F formation in muscle. Am J Physiol Endocrinol Metab. 2007;293:E1615–21.
19. Greiwe JS, Kwon G, McDaniel ML, Semenkovich CF. Leucine and insulin activate p70 S6 kinase through different pathways in human skeletal muscle. Am J Physiol Endocrinol Metab. 2001;281:E466–71.
20. Yoshizawa F, Tonouchi A, Miura Y, Yagasaki K, Funabiki R. Insulin-stimulated polypeptide chain elongation in the soleus muscle of mice. Biosci Biotechnol Biochem. 1995;59:348–9.
21. Bos C, Juillet B, Fouillet H, Turlan L, Daré S, Luengo C, N'tounda R, Benamouzig R, Gausserès N, et al. Postprandial metabolic utilization of wheat protein in humans. Am J Clin Nutr. 2005;81:87–94.
22. Boirie Y, Dangin M, Gachon P, Vasson MP, Maubois JL, Beaufrère B. Slow and fast dietary proteins differently modulate postprandial protein accretion. Proc Natl Acad Sci USA. 1997;94:14930–5.
23. de Castro JM. Circadian rhythms of the spontaneous meal pattern, macronutrient intake, and mood of humans. Physiol Behav. 1987;40:437–46.
24. Arnal MA, Mosoni L, Boirie Y, Houlier ML, Morin L, Verdier E, Ritz P, Antoine JM, Prugnaud J, et al. Protein pulse feeding improves protein retention in elderly women. Am J Clin Nutr. 1999;69:1202–8.
25. Reeves PG. Components of the AIN-93 diets as improvements in the AIN-76A diet. J Nutr. 1997;127 Suppl 5:S838–41.