

# Meal Distribution of Dietary Protein and Leucine Influences Long-Term Muscle Mass and Body Composition in Adult Rats<sup>1–3</sup>

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## Abstract

**Background:** Protein quantity and quality at a meal affect muscle protein synthesis (MPS); however, long-term effects of protein distribution at individual meals on adult muscle mass remain unknown.

**Objective:** We used a precise feeding protocol in adult rats to determine if optimizing postmeal MPS response by modifying the meal distribution of protein, and the amino acid leucine (Leu), would affect muscle mass.

**Methods:** Two studies were conducted with the use of male Sprague-Dawley rats (~300 g) trained to consume 3 meals/d, then assigned to diet treatments with identical macronutrient contents (16% of energy from protein, 54% from carbohydrates, and 30% from fat) but differing in protein quality or meal distribution. Study 1 provided 16% protein at each meal with the use of whey, egg white, soy, or wheat gluten, with Leu concentrations of 10.9%, 8.8%, 7.7%, and 6.8% (wt:wt), respectively. Study 2 used whey protein with 16% protein at each meal [balanced distribution (BD)] or meals with 8%, 8%, and 27% protein [unbalanced distribution (UD)]. MPS and translation factors 4E binding protein 1 (4E-BP1) and ribosomal protein p70S6 (S6K) were determined before and after breakfast meals at 2 and 11 wk. Muscle weights and body composition were measured at 11 wk.

**Results:** In study 1, the breakfast meal increased MPS and S6K in whey and egg treatments but not in wheat or soy treatments. Gastrocnemius weight was greater in the whey group (2.20 ± 0.03 g) than the soy group (1.95 ± 0.04 g) ( $P < 0.05$ ) and was intermediate in the egg and wheat groups. The wheat group had >20% more body fat than the soy, egg, or whey groups ( $P < 0.05$ ). Study 2, postmeal MPS and translation factors were 30–45% greater in the BD group than the UD group ( $P < 0.05$ ), resulting in 6% and 11% greater ( $P < 0.05$ ) gastrocnemius and soleus weights at 11 wk.

**Conclusion:** These studies show that meal distribution of protein and Leu influences MPS and long-term changes in adult muscle mass. *J Nutr* doi: 10.3945/jn.116.231779.

**Keywords:** muscle protein synthesis, initiation factors, whey protein, egg protein, soy protein

## Introduction

Dietary guidelines for protein are defined as net daily intake. However, evidence is accumulating that suggests that the meal distribution of daily protein is also important (1, 2). Older adults appear to require more indispensable amino acids (IAAs)<sup>6</sup> delivered as a bolus (i.e., grams per meal) to stimulate muscle protein synthesis (MPS) than do adults aged <25 y (3–7). Potential mechanisms to

explain the increased IAA need may include reduced blood flow to muscle tissues, reduced amino acid transporters at the cell membrane, and/or reduced anabolic signaling from growth factors or nutrients, including the amino acid leucine (Leu) (8).

Leu is an IAA known to stimulate the initiation of MPS through activation of mechanistic target of rapamycin complex 1 (mTORC1). Increases in intracellular Leu concentrations after a meal induce phosphorylation of mTORC1, resulting in the activation of the initiation factor 4F complex, assembly of the 48S initiation complex, and activation of the S6 ribosomal protein. The activation of these factors collectively stimulates MPS (9, 10). Although the molecular roles of Leu in the activation of mTORC1 and the stimulation of MPS are well characterized, the importance of meal distribution of Leu to changes in muscle mass remains unclear.

A recent study provided evidence to support protein distribution to optimize MPS in humans (1); however, another study found no difference with the use of an equal distribution of

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<sup>3</sup> Supplemental Tables 1 and 2 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at <http://jn.nutrition.org>.

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<sup>6</sup> Abbreviations used: BD, balanced distribution; IAA, indispensable amino acid; MPS, muscle protein synthesis; mTORC1, mechanistic target of rapamycin complex 1; S6K, translation factor ribosomal protein p70S6 kinase; UD, unbalanced distribution; 4E-BP1, translation initiation factor 4E binding protein 1.

protein across all meals compared with a large dinner meal (11). Ultimately, interpreting short-term postmeal changes in MPS in humans requires the measurement of long-term changes in muscle mass. Studies in humans requiring the precise control of macronutrient intakes at every meal for prolonged periods to produce what are likely small muscle changes in adults will remain a challenging task.

For initial proof-of-concept, we conducted 2 studies by using a meal-feeding protocol with adult rats to determine if meal distribution of protein and Leu is important for optimizing MPS and ultimately muscle protein accretion. Study 1 was designed to determine if feeding meals with identical energy and macronutrient content but with the use of proteins with different Leu contents would produce sustained differences in MPS and ultimately changes in muscle mass. Study 2 examined the importance of meal patterns with protein consumed in either a balanced distribution (BD) or an unbalanced distribution (UD). The total daily energy and macronutrient intakes were identical for BD and UD patterns. Both studies lasted 11 wk and evaluated the effect on postmeal MPS and long-term muscle mass.

## Methods

### Animals and diets

Male Sprague-Dawley rats ( $275 \pm 10$  g; Harlan-Teklad) were maintained at 24°C with a 12-h light:dark cycle and free access to water. 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.

**Study 1.** Rats were trained for 6 d to consume their food in defined meals, with a 4-g meal at 0700, a 4-g meal at 1300, and a 6-g meal at 1800. All meals were consumed within 20 min and food cups removed. This meal pattern was designed to be similar to the eating behavior of adults in the United States and to mimic breakfast, lunch, and dinner meals in our previous clinical protocol (12). On day 7, rats were randomly assigned to 1 of 4 diet groups ( $n = 21$ – $25$ ) containing wheat, soy, egg, or whey proteins (Supplemental Table 1). Wheat protein was supplemented with lysine to the concentration of the whey protein, consistent with previous experiments (12, 13) (Supplemental Table 2). The wheat protein diet was used for initial meal training. Each of the diet treatments provided 16% of energy from protein, 54% from carbohydrates, and 30% from lipids. The 4-g meals provided 0.68 g protein, with 46, 54, 60, and 74 mg Leu in the wheat, soy, egg, and whey groups, respectively, and the 6-g meal provided 1.02 g protein, with 69, 76, 90, and 111 mg Leu, respectively. We previously reported that the Leu threshold for the activation of mTORC1 signaling and MPS in this age and size of animal was  $\sim 55$ – $60$  mg (12, 13).

After 2 wk of being fed the respective diet treatments, 50% of the rats in each treatment group were used for the determination of body composition by DXA and returned to the feeding protocol for the remainder of the study. The remaining rats in each group were used for the measurement of MPS and the translation factors 4E binding protein 1 (4E-BP1) and ribosomal protein p70S6 (S6K).

Rats were food deprived for 12 h and then MPS was determined in a baseline control group before the first meal (i.e., food-deprived baseline group) or for each of the treatment groups at 90 min after completion of the 0700 meal (referred to as the breakfast meal). After consumption of the meal, rats were killed, blood was collected, and the gastrocnemius and soleus muscles and liver were removed and weighed. Rats used for DXA at 2 wk were used for the second DXA at week 11 and for measurements of MPS and translation factors as described for both 2 and 11 wk.

**Study 2.** All of the rats were trained to the same feeding protocol as in study 1 for 6 d with the use of the whey protein diet. On day 7, rats were randomly assigned to either a BD of protein identical to the whey group in study 1 (Supplemental Table 1) or to a UD ( $n = 21$ – $25$ ). BD treatment provided

16% of energy from protein at each meal, whereas UD treatment provided 8%, 8%, and 27.5% of energy from protein at breakfast, lunch, and dinner, respectively, by substituting protein or cornstarch in the whey protein diet. The Leu contents of the meals in the BD were 74, 74, and 111 mg, respectively, and the UD pattern was 38, 38, and 184 mg, respectively. Total daily energy, protein, IAA, carbohydrate, lipid, and fiber intakes were identical for both groups. Rats were examined after 2 and 11 wk, similar to study 1. The test meals for MPS were the habitual breakfast meals.

### Determination of MPS

Protein synthesis was measured in skeletal muscle 90 min after the breakfast meals at 2 and 11 wk by using the flooding dose method (14). A 100% enriched L-[ $^2$ H $_5$ ]-phenylalanine solution (150 mmol/L; Cambridge Isotope Laboratories) was administered at 150  $\mu$ mol/100 g body weight and injected via the tail vein (1 mL/100 g body weight) 80 min after completion of the meals. At 10 min after isotope injection (90 min postmeal), rats were killed by decapitation, and hind limbs were quickly removed and immersed in an ice-water mixture. Gastrocnemius muscle was removed from cooled hind limbs, frozen in liquid nitrogen, and stored at  $-80^\circ\text{C}$ .

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 gas chromatography–mass spectrometry, as described previously (12).

The muscle supernatant was used for the determination of intracellular free phenylalanine enrichment. Free amino acids were purified by ion exchange resin solid-phase extraction by using an EZfaast amino acid analysis sample testing kit (Phenomenex), and  $^2$ H $_5$ -phenylalanine enrichment was determined by using a propyl chloroformate derivative with gas chromatography–mass spectrometry by monitoring the ions at  $m/z$  206 ( $m$ ) and 211 ( $m + 5$ ) (15). Fractional rates of protein synthesis were determined from the rate of incorporation of L-[ $^2$ H $_5$ ]-phenylalanine into total mixed muscle protein as described previously (12).

### Plasma measurements

Plasma was obtained from trunk blood by centrifugation at  $1800 \times g$  for 10 min at 4°C. Plasma insulin concentrations were analyzed by using a commercial RIA kit for rat insulin (Linco Research). Plasma amino acid concentrations were analyzed by HPLC by using a Waters 2475 fluorescence detector (16).

### Phosphorylation of 4E-BP1 and S6K

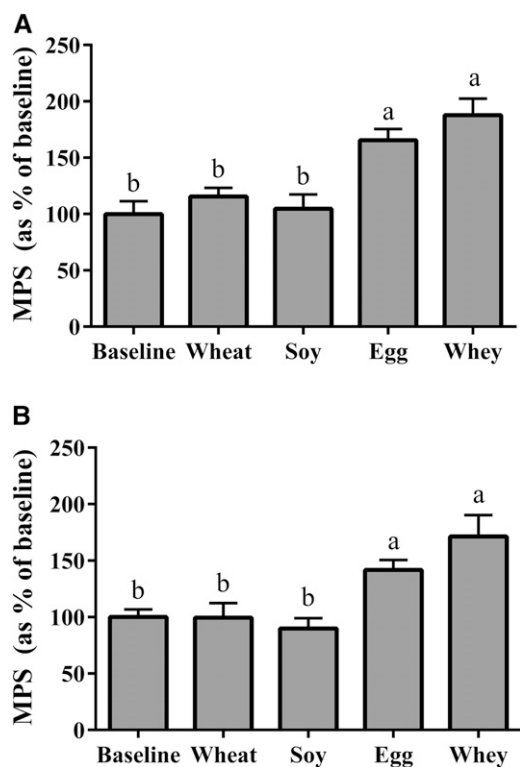
Muscle supernatants were subjected to protein immunoblot analysis as described previously (17) with the use of a rabbit polyclonal antibody to 4E-BP1 (Bethyl Labs) and a rabbit polyclonal S6K antibody (Bethyl Labs).

### Body composition

Before random assignment to treatments and at 2 and 11 wk, total body composition was assessed by using DXA analysis. Rats received an anesthetic by intraperitoneal injection of 0.3 mg dexmedetomidine/kg (Dexdomitor; Pfizer) for sedation during testing. The DXA instrument (QDR 4500A, software version 11.1.3; Hologic) was calibrated for rodent size as per the manufacturer's guidelines. Sedated rats were placed in the DXA and whole-body scans were performed. Scans for each time point were performed over 2 consecutive days with rats randomly assigned to order. All of the scans were analyzed by the same research technician who was blinded to group identity.

### Statistical analysis

All of the data were analyzed by SPSS 15.0 software package for windows. A 1-factor ANOVA was performed with the treatment group as the independent variable. When a significant overall effect was detected, differences between individual means were assessed by using Fisher's least significant difference post hoc test. Data sets were tested for normal distribution and variance homogeneity by using Levene's test. When variances were not homogeneous, means were compared by using a Games-Howell test. Correlations were determined by linear regression (Pearson's correlation).  $P < 0.05$  was considered significant. Values  $0.05 < P < 0.1$  were considered to be a trend. All values are presented as means  $\pm$  SEMs.



**FIGURE 1** MPS in rats fed diets containing wheat, soy, egg or whey proteins for 2 wk (A) or 11 wk (B) (study 1). Baseline values were  $2.12\% \pm 0.24\%/d$  (2 wk) and  $2.28\% \pm 0.17\%/d$  (11 wk) and were obtained in rats deprived of food for 12 h. MPS was determined at 90 min after the test meal. Values are means  $\pm$  SEMs,  $n = 5-8$ . Bars without a common letter differ,  $P < 0.05$ . MPS, muscle protein synthesis.

## Results

**Study 1.** MPS after the 4-g meal was significantly greater in the egg and whey groups, which did not differ, than in the wheat, soy, or baseline groups at both 2 wk (Figure 1A) and 11 wk (Figure 1B). The wheat, soy, and baseline groups did not differ from one another at either time. Each of the test meals provided 0.68 g

protein, but the proteins differed in IAA concentrations (Table 1), including the targeted differences for Leu in the wheat (46 mg), soy (54 mg), egg (60 mg), and whey (74 mg) protein diets.

Postprandial changes in plasma amino acid concentrations were consistent with the IAA concentration of the proteins (Table 1). Postmeal plasma Leu concentration increased from baseline in the whey protein group and the increase was greater than in the wheat and soy groups. The increase in plasma Leu concentration in the egg group was intermediate but not significantly different from the others. After the 4-g meal, plasma concentrations of isoleucine (Ile) and valine (Val) increased from baseline in the whey and egg groups but not in the wheat and soy groups. The wheat and soy groups were not different from baseline. For lysine (Lys), the highest dietary amounts were in the whey and Lys-supplemented wheat groups, and those amounts were reflected in the postprandial plasma concentrations. For methionine (Met), the highest content was in the egg protein meal and the lowest was in the soy protein meal, and those differences were apparent in the plasma changes. For threonine (Thr), the highest protein content was in the whey meal and the lowest was in the wheat meal, and those differences were also reflected in the plasma response to the meal.

Postprandial insulin concentrations at 90 min after the meal were not different between the groups or different from baseline at 2 wk (data not shown). At 11 wk, the wheat group had greater postprandial insulin at 90 min after feeding ( $296 \pm 60$  pmol/L) than did the egg ( $150 \pm 37$  pmol/L) or whey ( $132 \pm 26$  pmol/L) groups ( $P < 0.05$ ). The 90-min postprandial insulin concentrations were below initial baseline values for the egg and whey groups but not different from the soy group.

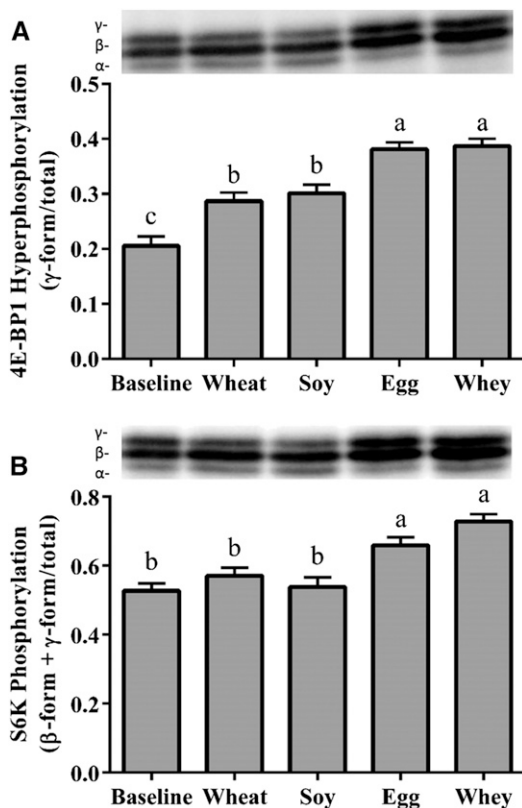
Changes in the plasma concentrations of Leu and MPS responses at 2 wk were consistent with the hyperphosphorylation of S6K (Figure 2B), with activation in the whey and egg groups greater than in wheat and soy groups and at baseline ( $P < 0.05$ ). Hyperphosphorylation of 4E-BP1 was intermediate for the wheat and soy groups and differed from baseline and the egg and whey groups ( $P < 0.05$ ) (Figure 2A). Translation factor responses at 11 wk were the same as those observed at 2 wk (data not shown).

**TABLE 1** Postprandial plasma amino acid concentrations in adult rats fed meals containing different protein sources<sup>1</sup>

	Baseline <sup>2</sup>	Wheat	Soy	Egg	Whey
Leucine, $\mu\text{mol/L}$	$91 \pm 4^b$	$78 \pm 8^b$	$78 \pm 8^b$	$130 \pm 18^{a,b}$	$146 \pm 28^a$
Contents in meal, mg		(46)	(54)	(60)	(74)
Isoleucine, $\mu\text{mol/L}$	$74 \pm 6.5^b$	$65 \pm 4^b$	$74 \pm 7^b$	$117 \pm 15^{a,b}$	$121 \pm 24^a$
Contents in meal, mg		(20)	(33)	(36)	(42)
Valine, $\mu\text{mol/L}$	$130 \pm 3.4^b$	$97 \pm 10^c$	$118 \pm 12^{b,c}$	$251 \pm 33^a$	$182 \pm 49^{a,b}$
Contents in meal, mg		(31)	(32)	(46)	(41)
$\sum$ BCAAs, $\mu\text{mol/L}$	$295 \pm 12^b$	$245 \pm 16^c$	$270 \pm 26^{b,c}$	$498 \pm 66^a$	$457 \pm 97^a$
Contents in meal, mg		(97)	(119)	(142)	(157)
Lysine, $\mu\text{mol/L}$	$543 \pm 15^{a,b}$	$549 \pm 80^{a,b}$	$435 \pm 62^b$	$492 \pm 65^{a,b}$	$626 \pm 46^a$
Contents in meal, mg		(62)	(43)	(44)	(62)
Methionine, $\mu\text{mol/L}$	$53 \pm 2.6^{a,b}$	$41 \pm 5^b$	$38 \pm 2^b$	$83 \pm 12^a$	$60 \pm 19^{a,b}$
Contents in meal, mg		(13)	(9)	(26)	(14)
Threonine, $\mu\text{mol/L}$	$389 \pm 54^c$	$452 \pm 17^c$	$518 \pm 67^{b,c}$	$537 \pm 73^b$	$860 \pm 74^a$
Contents in meal, mg		(18)	(26)	(31)	(44)

<sup>1</sup> Values are means  $\pm$  SEMs unless other indicated,  $n = 5-8$ . Values in parenthesis are the amino acids provided by each of the proteins in the 4-g breakfast meal. Labeled means without a common superscript letter differ,  $P < 0.05$ .  $\sum$ , sum.

<sup>2</sup> Premeal control (12-h food-deprived).



**FIGURE 2** Phosphorylation status of translation factors 4E-BP1 (A) and S6K (B) in the gastrocnemius muscle of rats fed diets containing wheat, soy, egg, or whey proteins for 2 wk (study 1). Baseline values were obtained in rats deprived of food for 12 h. Values are means  $\pm$  SEMs,  $n = 5-8$ . Bars without a common letter differ,  $P < 0.05$ . S6K, ribosomal protein p70S6 kinase; 4E-BP1, 4E binding protein 1.

Although the diets had the same energy density and macronutrient profiles, the meal proteins produced differences in body weight and body composition (Table 2). After 11 wk, body weight was significantly different ( $P < 0.05$ ) between groups. Specifically, the wheat group weighed more than the egg, whey, and soy groups and the egg and whey groups weighed more than the soy group. The wheat group had significantly more body fat than the other 3 groups, which did not differ. Body weight, FFM, and gastrocnemius weight were significantly lower in the soy group than in the wheat, egg, and whey groups. The whey group had significantly higher gastrocnemius mass than the wheat and soy groups, with the egg group being intermediate but not different.

**TABLE 2** Body weight, body composition, and tissue weights for adult rats fed diets containing different protein sources for 11 wk<sup>1</sup>

	Treatment group			
	Wheat	Soy	Egg	Whey
Body weight, g	392 $\pm$ 2.2 <sup>a</sup>	368 $\pm$ 2.6 <sup>c</sup>	385 $\pm$ 2.4 <sup>b</sup>	385 $\pm$ 2.4 <sup>b</sup>
Body fat, <sup>2</sup> %	12.4 $\pm$ 0.5 <sup>a</sup>	10.2 $\pm$ 0.7 <sup>b</sup>	9.7 $\pm$ 1.4 <sup>b</sup>	9.6 $\pm$ 3.0 <sup>b</sup>
FFM, <sup>2</sup> g	344 $\pm$ 3.3 <sup>a</sup>	330 $\pm$ 2.2 <sup>b</sup>	341 $\pm$ 2.6 <sup>a</sup>	345 $\pm$ 3.5 <sup>a</sup>
Gastrocnemius muscle, g	2.07 $\pm$ 0.03 <sup>b</sup>	1.95 $\pm$ 0.04 <sup>c</sup>	2.12 $\pm$ 0.03 <sup>a,b</sup>	2.20 $\pm$ 0.03 <sup>a</sup>
Soleus muscle, g	0.148 $\pm$ 0.004 <sup>a</sup>	0.135 $\pm$ 0.005 <sup>b</sup>	0.143 $\pm$ 0.003 <sup>a</sup>	0.149 $\pm$ 0.005 <sup>a</sup>
Liver, g	9.1 $\pm$ 0.4 <sup>a,b</sup>	8.6 $\pm$ 0.2 <sup>b</sup>	8.9 $\pm$ 0.3 <sup>a,b</sup>	9.4 $\pm$ 0.3 <sup>a</sup>

<sup>1</sup> Values are means  $\pm$  SEMs,  $n = 8-13$ . Labeled means without a common superscript letter differ,  $P < 0.05$ .

<sup>2</sup> Composition determined by DXA.

**Study 2.** Daily protein (2.38 g) and Leu (260 mg) intakes were the same in the BD and UD groups; however, the breakfast meal in the BD group contained 16% of energy as protein with 74 mg Leu, whereas the UD contained 8% protein and 38 mg Leu. Postprandial plasma IAA concentrations at 90 min after the breakfast meal were  $\sim 50\%$  higher in the BD group than in the UD group, with the differences significant for Leu, Val, and Thr but not for the others ( $P = 0.06-0.09$ ) (BD/UD  $\times 100$ : Leu, 187%; Ile, 168%; Val, 194%; Lys, 149%; Met, 146%; and Thr, 158%). Plasma concentrations of Val, Lys, and Met were below baseline values in the UD group at 90 min postmeal.

Postprandial MPS was significantly greater in the BD group than in the baseline and UD groups at 2 wk. The UD group was intermediate between the BD and baseline groups, with the trend nearing significance at 11 wk ( $P = 0.06$ ) (Figure 3A, B). Consistent with the MPS responses, phosphorylation of 4E-BP1 and S6K was significantly greater in the BD group compared with baseline (Figure 4). Phosphorylation of 4E-BP1 was intermediate in the UD group.

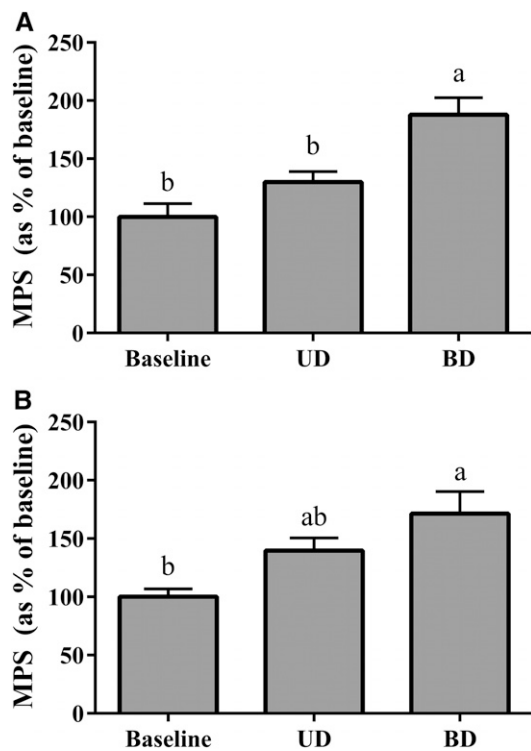
Body weight was significantly greater in the BD group (3.4%;  $P < 0.05$ ) than in the UD group at 11 wk, with a trend for greater total FFM (2.4%;  $P = 0.07$ ) (Table 3). Muscle weights in the BD group were 6.3% and 11.2% greater ( $P < 0.05$ ) than in the UD group for the gastrocnemius and soleus muscles, respectively. Fat mass did not differ between UD and BD groups.

## Discussion

Numerous studies have shown that the amount of protein or IAAs at a meal affects postprandial MPS and that older adults require more protein to maximize MPS than do younger adults, leading many to recommend meal-based guidelines for dietary protein (8, 18, 19). Although meal effects of protein on MPS are well established, long-term benefits of meal distribution of dietary protein and specifically the relation of postprandial MPS to changes in adult muscle mass remain controversial (1, 2, 11). The current studies used an animal model to evaluate the hypothesis that the meal distribution of dietary protein affects muscle mass. These studies show that optimizing daily protein in the first meal of the day to stimulate postmeal MPS, either by increasing protein quality or by redistributing protein from the dinner meal, resulted in greater muscle mass in adult rats. These studies also support the hypothesis that dietary Leu is an important component of protein quality in defining a minimum meal threshold for protein necessary to stimulate the translation initiation of MPS.

MPS responds to the amount of protein and, at least in part, the Leu content of the meal (12, 13, 20, 21). Furthermore, the





**FIGURE 3** MPS in rats fed meals with a BD or UD of whey protein for 2 wk (A) or 11 wk (B) (study 2). Baseline values were obtained in rats deprived of food for 12 h. Values are means  $\pm$  SEMs,  $n = 8-13$ . Bars without a common letter differ,  $P < 0.05$ . BD, balanced distribution; MPS, muscle protein synthesis; UD, unbalanced distribution.

maximum MPS postmeal response appears to reach a plateau with increasing protein (12, 22) or Leu (23). This “S-shaped” response curve of MPS combined with the finite duration of the postmeal anabolic response (12, 24, 25) led researchers to propose that distributing protein and/or Leu into multiple daily meals that each stimulate MPS should be superior to a single large meal for protein anabolism and muscle mass (8, 18).

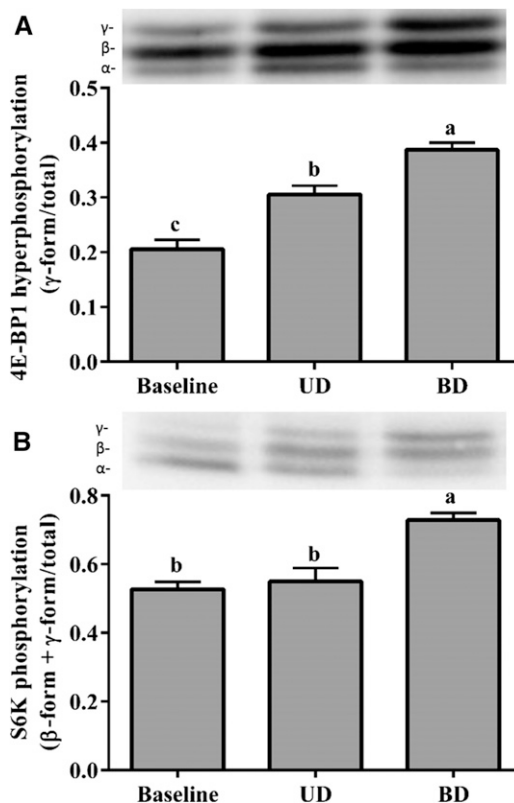
The current research tests this proposal with 2 studies that used a 3-meal/d feeding protocol in adult rats and showed that differences in protein distribution and the IAA content at individual meals influence muscle mass. Study 2 compared 2 feeding patterns with identical daily macronutrient compositions but with whey protein distributed in either a BD or a UD. The BD produced greater postprandial MPS after the breakfast meal and resulted in greater muscle mass after 11 wk.

Although the muscle mass of the 2 hind-limb muscles was greater in the BD group than in the UD group, the difference in total FFM was not significant (2.4%;  $P = 0.07$ ). The difference between individual muscle weights and FFM may be due to differential responses of other muscles or the difficulty in interpreting DXA data. We did not measure the chemical composition of the tissues and cannot rule out changes in hydration or glycogen content between groups; however, we speculate that this is unlikely in the diets containing identical macronutrient contents and with the meal patterns, physical activity, and water intakes being consistent across the groups for 3 mo. An alternative possibility is that the meal distribution of protein affects organ size. We found that the UD group had greater liver mass (7.4%;  $P < 0.05$ ), presumably associated with the greater influx of amino acids and amino-nitrogen at the dinner meal (26). Other organs that may be influenced by

protein content of the meal include the kidneys and gastrointestinal tract.

A limitation of this study is that MPS was not measured after the dinner meal. Previous research from our laboratory (12) and others (22) shows that the meal response of MPS reaches a plateau with increasing protein amounts, and certainly the mTORC1 initiation signal reaches a Leu plateau (23). For the specific age and size of rats in this experiment, and within the limits of our isotope measurements, we cannot detect differences in MPS between groups at dietary intakes  $>100$  mg Leu (12, 13), leading us to assume that MPS was not different between groups after the dinner meal. Furthermore, the protein differences in the breakfast meals produced differences in MPS and muscle mass that were not fully compensated by balancing total daily intake with the larger dinner meal (containing 184 mg Leu compared with 111 mg for the UD and BD, respectively). Similar MPS effects of meal distribution were reported in a clinical trial in which BD resulted in greater net MPS for the 24-h period (1).

Study 1 provides evidence that modifying the meal composition of IAAs, specifically Leu, affects both MPS and muscle mass. This study used meals with identical macronutrient compositions but differing in food sources of proteins and the associated amino acid compositions. The proteins were selected for differences in Leu content. Although the concentrations of each of the IAAs differed among the proteins, the 16%-protein diets exceeded the NRC requirements for each of the IAAs for adult rats (27) (Note: wheat gluten does not meet the NRC requirement for Lys and was supplemented to the whey protein



**FIGURE 4** Phosphorylation status of 4E-BP1 (A) and S6K (B) in the gastrocnemius muscle of rats fed meals with a BD or UD of whey protein for 11 wk (study 2). Baseline values were obtained in rats deprived of food for 12 h. Values are means  $\pm$  SEMs,  $n = 8-13$ . Bars without a common letter differ,  $P < 0.05$ . BD, balanced distribution; S6K, ribosomal protein p70S6 kinase; UD, unbalanced distribution; 4E-BP1, 4E binding protein 1.

**TABLE 3** Body weight, body composition, and tissue weights of adult rats fed diets with whey protein distributed in 3 BD or UD meals for 11 wk<sup>1</sup>

	Meal treatment	
	UD	BD
Body weight, g	372 ± 2.1	385 ± 2.4*
Body fat, <sup>2</sup> %	9.7 ± 1.9	9.6 ± 3.0
FFM, <sup>2</sup> g	337 ± 2.5	345 ± 3.5 <sup>†</sup>
Gastrocnemius muscle, g	2.07 ± 0.05	2.20 ± 0.03*
Soleus muscle, g	0.134 ± 0.005	0.149 ± 0.005*
Liver, g	10.1 ± 0.4	9.4 ± 0.3*

<sup>1</sup> Values are means ± SEMs,  $n = 8-13$ . \*Significant difference,  $P < 0.05$ ; <sup>†</sup>trend for difference,  $P = 0.08$ . BD, balanced distribution; UD, unbalanced distribution.

<sup>2</sup> Determined by DXA.

content.) Consistent with our previous reports (12, 13), this study shows a threshold response of MPS to the Leu content of the meal.

The importance of Leu as a dietary signal to optimize MPS has been shown with meals containing insufficient protein or IAAs in studies in humans (20, 28, 29) and animals (13, 21, 30). These studies examined MPS after a meal with suboptimal amounts of protein or IAAs and found that supplementing Leu to the meal would initiate the postprandial MPS response. Likewise, animal studies by Crozier et al. (23) and Norton et al. (12) showed that the meal response of MPS relates to Leu intake and hyperphosphorylation of translation factors 4E-BP1 and S6K. Consistent with these reports, 4E-BP1 in study 1 appeared to respond to the Leu intake, with phosphorylation in the wheat and soy groups being intermediate between baseline and the egg and whey groups ( $P < 0.05$ ); and in study 2, the phosphorylation of 4E-BP1 in the UD was intermediate between baseline and the BD group ( $P < 0.05$ ). The meal response of S6K phosphorylation was somewhat different, with the wheat and soy groups not different from baseline in study 1 and the UD group not different from baseline in study 2. The relative importance of 4E-BP1 and S6K in postprandial MPS response is unknown; however, in these studies the S6K responses parallel changes in MPS.

The strengths of these studies are the precise, long-term control of meal patterns and food intake, showing that meal distribution of protein can affect muscle mass in adult rats. There is increasing evidence that older adults require additional protein to optimize postprandial MPS. However, the critical evidence that increasing protein quantity or quality to stimulate MPS ultimately produces positive changes in muscle mass is difficult to establish in free-living adults in whom changes in muscle mass may be  $<1\%/y$  (31). There are reports that protein distribution can minimize muscle loss during acute catabolic conditions, such as bed rest (31) or weight loss (32), but showing age-related changes in healthy adults will require prolonged studies with precise diet and activity controls that will be difficult to execute.

Limitations of this research are inherent in our attempt to extrapolate postmeal MPS measurements to predict changes in muscle mass. Tissue weight changes result from the ever-changing balance between protein synthesis and breakdown. Projecting long-term changes in muscle mass on the basis of a single, short-term, postprandial measure of MPS is obviously not a quantitative relation (33), and current methods are insufficient to monitor minute-to-minute changes in both synthesis and breakdown. Still, the primary outcomes of this study show

that the meal distribution of protein can affect adult muscle mass and that MPS provides a tool for evaluating optimal protein quantity and quality for skeletal muscles.

The extrapolation of these findings in rats to humans requires caution. We attempted to model human meal patterns that we observed in our long-term weight-management studies (34). We assume that the magnitude of the changes observed in rats is likely greater than in humans, but we have no reason to suspect that the direction of change differs. Although this study provides proof-of-concept, long-term benefits of meal distribution of dietary protein remain to be established in a human study.

In summary, these studies used a meal-feeding protocol in adult rats to evaluate the relation of MPS to protein quantity and distribution and long-term changes in muscle mass. Our findings support the use of postprandial MPS response as a biomarker for assessing dietary protein needs to optimize skeletal muscle mass and that multiple daily meals that increase MPS are superior to a single large meal response in rats. Further studies will need to be performed to confirm the applicability to humans. The study also supports the hypothesis that dietary Leu is an important component of protein quality in defining a minimum meal threshold for protein necessary to stimulate translation initiation of MPS.

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### References

- Mamerow MM, Mettler JA, English KL, Casperson SL, Arentson-Lantz E, Sheffield-Moore M, Layman DK, Paddon-Jones D. Dietary protein distribution positively influences 23-h muscle protein synthesis in healthy adults. *J Nutr* 2014;144:876-80.
- Murphy CH, Churchward-Venne TA, Mitchell CJ, Kolar NM, Kassis A, Karagounis LG, Burke LM, Hawley JA, Phillips SM. Hypoenergetic diet-induced reductions in myofibrillar protein synthesis are restored with resistance training and balanced daily protein ingestion in older men. *Am J Physiol Endocrinol Metab* 2015;308:E734-43.
- Volpi E, Kobayashi H, Sheffield-Moore M, Mittendorfer B, Wolfe RR. Essential amino acids are primarily responsible for the amino acid stimulation of muscle protein anabolism in healthy elderly adults. *Am J Clin Nutr* 2003;78:250-8.
- Katsanos CS, Kobayashi H, Sheffield-Moore M, Aarsland A, Wolfe RR. Aging is associated with diminished accretion of muscle proteins after the ingestion of a small bolus of essential amino acids. *Am J Clin Nutr* 2005;82:1065-73.
- Symons TB, Schutzler SE, Cocke TL, Chinkes DL, Wolfe RR, Paddon-Jones D. Aging does not impair the anabolic response to a protein-rich meal. *Am J Clin Nutr* 2007;86:451-6.
- Pennings B, Groen B, de Lange A, Gijzen AP, Zorenc AH, Senden JMG, van Loon LJC. Amino acid absorption and subsequent muscle protein accretion following graded intakes of whey protein in elderly men. *Am J Physiol Endocrinol Metab* 2012;302:E992-9.
- Moore DR, Churchward-Venne TA, Witard O, Breen L, Burd NA, Tipton KD, Phillips SM. Protein ingestion to stimulate myofibrillar protein synthesis requires greater relative protein intakes in healthy older versus younger men. *J Gerontol A Biol Sci Med Sci* 2015;70:57-62.
- Layman DK, Anthony TG, Rasmussen BB, Adams SH, Lynch CJ, Brinkworth GD, Davis TA. Defining meal requirements for protein to optimize metabolic roles of amino acids. *Am J Clin Nutr* 2015;101 (Suppl):1330S-8S.

9. Kimball SR, Jefferson LS. Signaling pathways and molecular mechanisms through which branched-chain amino acids mediate translational control of protein synthesis. *J Nutr* 2006;136(Suppl):227S–31S.
10. Bar-Peled L, Sabatini DM. Regulation of mTORC1 by amino acids. *Trends Cell Biol* 2014;24:400–6.
11. Kim I-Y, Schutzler S, Schrader A, Spencer H, Kortebein P, Deutz NEP, Wolfe RR, Ferrando A. Quantity of dietary protein intake, but not pattern of intake, affects net protein balance primarily through differences in protein synthesis in older adults. *Am J Physiol Endocrinol Metab* 2015;308:E21–8.
12. Norton LE, Layman DK, Bunpo P, Anthony TG, Brana DV, Garlick PJ. 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. *J Nutr* 2009;139:1103–9.
13. Norton LE, Wilson GJ, Layman DK, Moulton CJ, Garlick PJ. Leucine content of dietary proteins is determinant of postprandial skeletal muscle protein synthesis in adult rats. *Nutr Metab (Lond)* 2012;9:67–76.
14. Garlick PJ, McNurlan MA, Preedy VR. A rapid and convenient technique for measuring the rate of protein synthesis in tissues by injection of [<sup>3</sup>H]phenylalanine. *Biochem J* 1980;192:719–23.
15. McNurlan MA, Essen P, Thorell A, Calder AG, Anderson SE, Ljungqvist O, Sandgren A, Grant I, Tjader I, Ballmer PE. Response of protein synthesis in human skeletal muscle to insulin: an investigation with L-[2H<sup>5</sup>]phenylalanine. *Am J Physiol* 1994;267:E102–8.
16. 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.
17. Anthony TG, McDaniel GJ, Knoll P, Bunpo P, Paul GL, McNurlan MA. 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.
18. Paddon-Jones D, Rasmussen BB. Dietary protein recommendations and the prevention of sarcopenia. *Curr Opin Clin Nutr Metab Care* 2009;12:86–90.
19. Bauer J, Biolo G, Cederholm T, Cesari M, Cruz-Jentoff AJ, Morley JE, Phillips S, Sieber C, Stehle P, Teta D, et al. Evidence-based recommendations for optimal dietary protein intake in older people: a position paper from the PROT-AGE study group. *J Am Med Dir Assoc* 2013;14:542–59.
20. Katsanos CS, Kobayashi H, Sheffield-Moore M, Aarsland A, Wolfe RR. A high proportion of leucine is required for optimal stimulation of the rate of muscle protein synthesis by essential amino acids in the elderly. *Am J Physiol Endocrinol Metab* 2006;291:E381–7.
21. Dardevet D, Sornet C, Bayle G, Prugnaud J, Pouyet C, Grizard J. Postprandial stimulation of muscle protein synthesis in old rats can be restored by a leucine-supplemented meal. *J Nutr* 2002;132:95–100.
22. Moore DR, Robinson MJ, Fry JL, Tang JE, Glover EI, Wilkinson SB, Prior T, Tarnopolsky MA, Phillips SM. Ingested protein dose response of muscle and albumin protein synthesis after resistance exercise in young men. *Am J Clin Nutr* 2009;89:161–8.
23. 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.
24. Bohe J, Aili 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;532:575–9.
25. Atherton PJ, Etheridge T, Watt PW, Wilkinson D, Selby A, Rankin D, Smith K, Rennie MJ. Muscle full effect after oral protein: time-dependent concordance and discordance between human muscle protein synthesis and mTORC1 signaling. *Am J Clin Nutr* 2010;92:1080–8.
26. Ashida K, Harper AE. Metabolic adaptations in higher animals. VI. Liver arginase activity during adaptation to high protein diet. *Proc Soc Exp Biol Med* 1961;107:151–6.
27. National Research Council Committee on Animal Nutrition, Board on Agriculture. Nutrient requirements of laboratory animals. 4th ed. Washington (DC): National Academies Press; 1995.
28. Rieu I, Balage M, Sornet C, Giraudet C, Pujos E, Grizard J, Mosoni L, Dardevet D. Leucine supplementation improves muscle protein synthesis in elderly men independently of hyperaminoacidaemia. *J Physiol* 2006;575:305–15.
29. Churchward-Venne TA, Breen L, Di Donato DM, Hector AJ, Mitchell CJ, Moore DR, Stellingwerff T, Breuille D, Offord EA, Baker SK, et al. Leucine supplementation of a low-protein mixed macronutrient beverage enhances myofibrillar protein synthesis in young men: a double-blind randomized trial. *Am J Clin Nutr* 2014;99:276–86.
30. Torrazza RM, Suryawan A, Gazzaneo MC, Orellana RA, Frank JW, Nguyen HV, Fiorotto ML, El-Kadi S, Davis TA. Leucine supplementation of a low-protein meal increases skeletal muscle and visceral tissue protein synthesis in neonatal pigs by stimulating mTOR-dependent translation initiation. *J Nutr* 2010;140:2145–52.
31. English KL, Paddon-Jones D. Protecting muscle mass and function in older adults during bed rest. *Curr Opin Clin Nutr Metab Care* 2010;13:34–9.
32. Layman DK, Boileau RA, Erickson DJ, Painter JE, Shiue H, Sather C, Christou DD. A reduced ratio of dietary carbohydrate to protein improves body composition and blood lipid profiles during weight loss in adult women. *J Nutr* 2003;133:411–7.
33. Mitchell CJ, Churchward-Venne TA, Parise G, Bellamy K, Baker SK, Smith K, Atherton PJ, Phillips SM. Acute post-exercise myofibrillar protein synthesis is not correlated with resistance training-induced muscle hypertrophy in young men. *PLoS One* 2014;9:e89431.
34. Layman DK, Evans EM, Erickson D, Seyler J, Weber J, Bagshaw D, Griel A, Psota T, Kris-Etherton P. A moderate-protein diet produces sustained weight loss and long-term changes in body composition and blood lipids in obese adults. *J Nutr* 2009;139:514–21.