Ingested protein dose response of muscle and albumin protein synthesis after resistance exercise in young men[1–3]

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ABSTRACT

Background: The anabolic effect of resistance exercise is enhanced by the provision of dietary protein.

Objectives: We aimed to determine the ingested protein dose response of muscle (MPS) and albumin protein synthesis (APS) after resistance exercise. In addition, we measured the phosphorylation of candidate signaling proteins thought to regulate acute changes in MPS.

Design: Six healthy young men reported to the laboratory on 5 separate occasions to perform an intense bout of leg-based resistance exercise. After exercise, participants consumed, in a randomized order, drinks containing 0, 5, 10, 20, or 40 g whole egg protein. Protein synthesis and whole-body leucine oxidation were measured over 4 h after exercise by a primed constant infusion of [1-^{13}C]leucine.

Results: MPS displayed a dose response to dietary protein ingestion and was maximally stimulated at 20 g. The phosphorylation of ribosomal protein S6 kinase (Thr^{389}), ribosomal protein S6 ($Ser^{240/244}$), and the ε-subunit of eukaryotic initiation factor 2B (Ser^{539}) were unaffected by protein ingestion. APS increased in a dose-dependent manner and also reached a plateau at 20 g ingested protein. Leucine oxidation was significantly increased after 20 and 40 g protein were ingested.

Conclusions: Ingestion of 20 g intact protein is sufficient to maximally stimulate MPS and APS after resistance exercise. Phosphorylation of candidate signaling proteins was not enhanced with any dose of protein ingested, which suggested that the stimulation of MPS after resistance exercise may be related to amino acid availability. Finally, dietary protein consumed after exercise in excess of the rate at which it can be incorporated into tissue protein stimulates irreversible oxidation. *Am J Clin Nutr* 2009;89:161–8.

INTRODUCTION

The provision of an exogenous source of amino acids, likely only the essential amino acids (EAAs), enhances the anabolic effect of resistance exercise on muscle (1–4). Muscle protein synthesis (MPS), not breakdown, is the more responsive variable to a variety of anabolic stimuli in healthy individuals (5) and is stimulated in a dose-dependent manner with increasing amounts of ingested EAAs at rest (6, 7). Data from a series of studies suggest that a similar dose response of protein synthesis to amino acids exists after resistance exercise (2, 8, 9). No study to date has directly measured a dose-response relation between ingested whole protein and muscle protein synthetic rates after resistance exercise.

The acute synthesis of muscle proteins is primarily regulated at the level of messenger RNA translation via the activation of a variety of intracellular signaling proteins, especially those of the mammalian target of rapamycin (mTOR) signaling cascade (10). Stimulation of MPS in humans after feeding or resistance exercise is accompanied by enhanced phosphorylation, and presumably activity, of the mTOR signaling pathway, including the 70-kDa ribosomal protein S6 kinase (S6K1) and its target ribosomal protein S6 (rpS6) (7, 11–16). In addition, global rates of protein synthesis are tightly regulated by the activity of the guanine nucleotide exchange factor eukaryotic initiation factor 2B (eIF2B), which is responsive to both amino acids and exercise and is under the control of glycogen synthase kinase-3β in rats (17, 18) and cultured myoblasts (19). Collectively, these observations suggest that changes in MPS in response to feeding and exercise may be regulated by specific downstream target proteins of mTOR signaling such as S6K1, rpS6, and eIF2B.

Albumin is a major hepatic-derived plasma protein that is unaffected by resistance exercise in young men (20). However, albumin synthesis is stimulated by an increased availability of amino acids (21–23). It has been suggested that dietary amino acids may be incorporated into albumin protein in an effort to minimize their irreversible oxidation (21). Thus, a feeding-induced increase in albumin protein synthesis may serve as a mechanism to ''store'' excess amino acids from the diet until they are needed during periods of reduced supply (21).

The present study was designed to yield a dose-response relation for ingested protein in the stimulation of muscle and albumin protein synthesis after resistance exercise. We also wished

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to characterize other fates of amino acids when the amount of protein ingested might exceed the capacity for amino acids to be used in MPS, namely irreversible oxidation. We hypothesized that mixed MPS would demonstrate a dose response to dietary protein after resistance exercise and that the maximal effective (ie, maximally stimulatory for MPS) dose would be similar to what has previously been reported to be maximal at rest (7). In addition, we hypothesized that plasma albumin protein synthesis would display a similar dose response to dietary protein as mixed muscle protein. However, above an ingested dose of protein that maximally stimulated muscle and albumin protein synthesis, we hypothesized that amino acid oxidation would increase.

SUBJECT AND METHODS

Subjects

Six healthy active males (mean ± SE: 22 ± 2 y; 86.1 ± 7.6 kg; 1.82 ± 0.1 m) who had ≥4 mo of previous recreational weightlifting experience (range: 4 mo–8 y) volunteered to participate in the study. Our repeated-measures design led us to recruit participants with previous resistance training experience to minimize the occurrence of exercise-induced muscle damage that can occur with novel exercise in untrained individuals as well as to minimize any learning effects during trials. Participants were informed about the experimental procedure to be used as well as the purpose of the study and all potential risks before giving written consent. All participants were deemed healthy on the basis of their response to a routine medical screening questionnaire. The study conformed to all standards for the use of human subjects in research as outlined in the Helsinki declaration and was approved by the local Research Ethics Board of McMaster University and Hamilton Health Sciences.

Study design

Participants reported to the laboratory on 5 separate occasions separated by at least 1 wk. Each trial began with the participant performing an acute bout of intense leg resistance exercise. After exercise, participants consumed a drink containing 0, 5, 10, 20, or 40 g egg protein in a randomized order. Whole-body leucine oxidation as well as albumin protein synthesis (APS) and mixed MPS were measured using a primed constant infusion of [1-^{13}C]leucine over 4 h. Leucine was chosen as a tracer, because it is an essential amino acid that is primarily metabolized within the lean tissues of the body. Furthermore, muscle tissue is both enriched in branched-chain amino acids (BCAAs) such as leucine and is also a major site for leucine oxidation.

Diet

Before each trial, participants were supplied with prepackaged diets for 2 d that provided a moderate protein intake (1.4 g · kg^{-1} · d^{-1}) for resistance-trained athletes (24). Energy needs of the controlled diets were estimated according to the Harris-Benedict equation and were adjusted using a moderate activity factor (1.6) for all participants to account for habitual activity. Body mass did not change over the course of the controlled diet, suggesting participants were in energy balance. Participants were also required to complete diet records before the start of the study to provide an estimate of habitual macronutrient consumption as analyzed using a commercially available software program (Nutritionist V; First Data Bank, San Bruno, CA). Reference lists for portion size estimates were provided to participants who were instructed to record all food or drink consumed in a diet log during a 3-d period (ie, 2 weekdays and 1 weekend). On the basis of the responses, the average habitual protein intake was identical to the controlled diet (1.5 ± 0.2 compared with 1.4 ± 0.1 g · kg^{-1} · d^{-1}, respectively; $P = 0.8$, paired t test), whereas the energy intake was slightly less than the controlled diet (130 ± 10 compared with 170 ± 3 kJ · kg^{-1} · d^{-1}, respectively; $P < 0.05$, paired t test). This apparent discrepancy in energy intake between the controlled and the habitual diets is likely related to the underreporting of true energy intake commonly seen with self-reported dietary assessments (25). Nonetheless, because body mass did not change throughout the duration of the study, controlled diets were kept consistent to ensure individuals had identical macronutrient consumption for the 2 d before each trial.

Infusion protocol

Participants reported to the laboratory at 0700 after an overnight fast, having refrained from all resistance exercise and any strenuous physical activity for at least 3 d. Bilateral resistance exercise was performed on guided-motion machines and involved 4 sets each of leg press, knee extension, and leg curl using a predetermined load designed to elicit failure within 8–10 repetitions. Each set was completed within ≈25 s with a rest period of 120 s between each set. After exercise, a baseline breath sample was collected for determination of $^{13}CO_2$ enrichment by isotope ratio mass spectrometry (BreathMat Plus; Finnigan MAT GmbH, Bremen, Germany). A polytetrafluoroethylene catheter was inserted in the medial vein of each arm, one for tracer infusion and the other for arterialized blood sampling. Arterialized blood samples were obtained by wrapping the forearm in a heating blanket for the duration of the infusion. Baseline blood samples were drawn, and then participants received priming doses of Na$H^{13}CO_2$ (2.35 μmol/kg) and [1-^{13}C]leucine (7.6 μmol/kg, 99 atom percent; Cambridge Isotopes, Andover, MA) before beginning a constant [1-^{13}C]leucine infusion (7.6 μmol · kg^{-1} · h^{-1}) (**Figure 1**). Immediately after the onset of the infusion, participants consumed a drink containing 0, 5, 10, 20, or 40 g whole-egg protein dissolved in 400 mL water. The amino acid content of the protein was (in percent content, wt:wt): Ala, 6.3; Arg, 5.7; Asp, 8.7; Cys, 2.4; Gln, 13.7; Gly, 3.6; His, 2.1; Ile, 5.9; Leu, 8.4; Lys, 5.9; Met, 3.8; Phe, 6; Pro, 3.6; Ser, 7.1; Thr, 4.3; Trp, 1.4; Tyr, 3.9; and Val, 7.2. On the basis of a leucine content of ≈8% in egg protein, drinks were enriched to 5% with [1-^{13}C]leucine to minimize disturbances in isotopic steady state. Arterialized blood samples were collected every 0.5–1 h into evacuated heparinized tubes and chilled on ice. Within 5 min of sampling, 100 μL whole blood was deproteinized with 0.6 mol/L perchloric acid (PCA) and the remaining sample was centrifuged (4000 *g*) for 5 min to separate plasma. PCA extracts and blood plasma were stored at −20°C for further analysis. Biopsy samples were taken from the vastus lateralis of a randomly selected thigh using a 5-mm Bergström needle (modified for manual suction) under 2% xylocaine local anesthesia. Muscle biopsies were freed from any visible blood, fat, and connective tissue and rapidly frozen in liquid nitrogen for further analysis. Muscle biopsies for a given trial were taken from separate

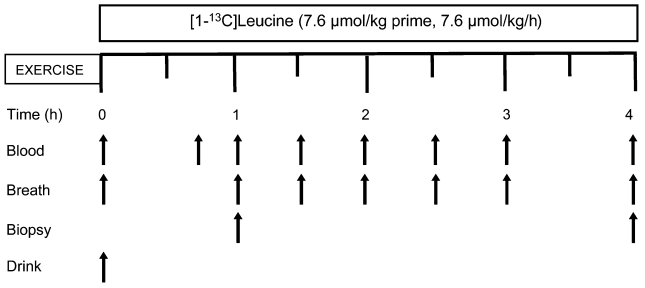


FIGURE 1. Schematic diagram of infusion protocol. Arrows correspond to the time at which blood, breath, or muscle biopsy samples were taken. A drink containing 0, 5, 10, 20, or 40 g protein was ingested in a randomized order immediately after a bout of resistance exercise.

incisions (≈4–5 cm apart) from the same leg at 1 and 4 h with alternate legs being sampled for each subsequent trial.

Analysis

Blood amino acid concentrations were measured from the PCA extract by HPLC as previously described (26). Plasma insulin concentration was determined by a standard radioimmunoassay kit (Coat-a-Count; Diagnostic Products, Los Angeles, CA). Blood glucose concentration was measured spectrophometrically using a standard glucose peroxidase enzymatic kit (Stanbio Laboratory, Boerne, TX). Plasma urea concentration was measured using a standard spectrophometric kit (Pointe Scientific Inc, Canton, MI). Plasma enrichment of the *t*-BDMS derivative of α-[^{13}C]-ketoisocaproate acid (α-KIC) was measured by gas chromatography–mass spectrometry (Hewlett-Packard 6890; MSD model 5973 Network; Agilent Technologies, Santa Clara, CA) as a surrogate for intramuscular (27, 28) and hepatic (29) leucyl-transfer RNA labeling.

Mixed muscle proteins were isolated from biopsy specimens (≈30 mg wet weight) by homogenizing with 10 μL/mg 100% acetonitrile. The samples were mixed by vortex for 10 min and then centrifuged (15,000 *g*) for 5 min. The resultant pellet was washed again with 10 μL/mg 100% acetonitrile, once with 1 mL double-distilled H_2O, and finally with 1 mL 95% ethanol. The pellet was lyophilized and amino acids were liberated from mixed muscle tissue protein by 6N HCl (400 μL/mg) acid hydrolysis at 110°C for 24 h. Free amino acids were purified using cation exchange chromatography (Dowex 50WX8-200 resin; Sigma-Aldrich Ltd, Oakville, Canada) and converted to their *N*-acetyl-n-propyl ester derivatives for analysis by gas chromatography (GC) combustion-isotope ratio mass spectrometry (GC model; Hewlett-Packard 6890; Agilent Technologies; IRMS model Delta Plus XP; Thermo Finnigan, Waltham, MA). Derivitized amino acids were separated on a 30-m DB-1701 column (temperature program: 110°C for 2 min; 10°C/min ramp to 190°C; hold for 2 min; 2°C/min ramp to 210°C; 20°C/min ramp to 280°C; hold for 5 min) before combustion.

To isolate intracellular proteins for Western blotting, a small piece of wet muscle (≈20 mg) was homogenized by hand on ice in a tris-buffered lysis buffer (pH 7.6) containing 0.1% (wt:vol) sodium dodecyl sulfate, 0.5% (wt:vol) deoxycholaic acid, 15 mmol/L tris-HCl, 167 mmol/L NaCl, and commercially available phosphatase and protease inhibitors (Roche Diagnostics, Indianapolis, IN). Protein content of the homogenates was determined by bicinchoninic acid protein assay (Thermo Scientific, Rockford, IL), and then samples (20 μg protein) were loaded on a 8–16% gradient sodium dodecyl sulfate polyacrylamide gel (Pierce, Rockford, IL) before being transferred to a PVDF membrane for blotting. Membranes were blocked with 5% (wt:vol) bovine serum albumin (rpS6, eIF2Bε, and actin) or 5% nonfat milk (S6K1) in tris-buffered saline with 0.1% Tween (vol:vol) (TBST) and then incubated overnight in primary antibody at 4°C: S6K1 Thr^{389} (catalog no 11759, 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA); rpS6 Ser$^{240/244}$ (2215, 1:4000; Cell Signaling, Danvers, MA); and eIF2Bε Ser^{539} (24775, 1:4000; Genetex, San Antonio, TX). We measured rpS6 Ser$^{240/244}$ phosphorylation, because this site is phosphorylated by S6K1 and is responsive to feeding after resistance exercise (16, 30). As a loading control, protein phosphorylation was normalized to total actin by incubating in primary antibody (A2066, 1:10,000; Sigma) for 1 h at room temperature. After washing in TBST, membranes were incubated in horseradish peroxidase-linked anti-rabbit immunoglobulin G secondary antibody (NA934V, 1:50,000; Amersham Biosciences, Piscataway, NJ), washed with TBST, and developed using Pierce's Supersignal West Dura HRP detection kit (Thermo Fisher Scientific, Nepean, Canada). Images were captured on a Fluorochem SP imaging system (Alpha Innotech, San Leandro, CA).

Fibrinogen was precipitated from plasma (≈1.5 mL) by adding 4 IU thrombin (Sigma-Aldrich Ltd) and 40 μL 1 mol/L $CaCl_2$ and incubating at 4°C for 12 h. Albumin was isolated from plasma by its differential solubility in ethanol using an assay adapted from a previously published method (31). Specifically, plasma proteins were precipitated from 500 μL fibrinogen-free plasma by adding 1 mL 10% trichloroacetic acid (wt:vol). After centrifugation at 4500 *g* for 5 min, the supernatant fluid was discarded and the resultant pellet was resuspended in 500 μL ddH_2O. Albumin was solubilized by adding 2.5 mL 1% TCA (wt:vol) in 100% ethanol and centrifuged at 4500 rpm for 5 min. The supernatant fluid was collected, and albumin was precipitated by adding 1 mL 26.8% ammonium sulfate (wt:vol). After centrifugation at 4500 rpm for 5 min, the supernatant fluid was removed and discarded and the resultant pellet was washed once more with 1 mL 26.8% ammonium sulfate. To remove any residual free amino acids, the albumin pellet was washed twice with 1 mL 0.2 mol/L PCA. Finally, the pellet was lyophilized and the bound amino acids were liberated by acid hydrolysis and analyzed using GC-combustion-isotope ratio mass spectrometry as described above. Plasma albumin concentration was determined spectrophometrically using a standard bromocresol green assay procedure (Pointe Scientific Inc, Canton, MI).

Calculations

The rates of mixed muscle and albumin protein synthesis were calculated using the standard precursor-product method:

$$\text{Fractional protein synthesis(FSR, \%/h)} = \Delta E_b / E_p \times 1/t \times 100 \quad (1)$$

where ΔE_b is the change in bound protein enrichment between 2 points, E_p is the mean enrichment over time of arterialized venous plasma α-KIC, and t is the time between biopsies (for mixed muscle FSR) or blood samples (for albumin FSR, t = 60 and 240 min). E_p was calculated as the area under the venous

plasma α-KIC enrichment by time curve divided by time. Leucine oxidation was calculated from the appearance of the ^{13}C-label in expired CO_2 using the reciprocal pool model with fractional bicarbonate retention factors of 0.7 and 0.83 for fasted (0 g protein) and fed (5–40 g protein) states, respectively (32, 33). The area under the leucine oxidation by time curve was calculated using Prism 3.0 graphing software (GraphPad Software Inc, San Diego, CA) as an estimate of total leucine oxidation. In addition, the area under the plasma insulin concentration by time curve was also calculated with the same graphing software.

Statistics

Because the present study used a within-subject design, changes in MPS, APS, and plasma albumin concentrations were analyzed using a one-factor (1 × 5; condition) repeated-measures analysis of variance (ANOVA). A 2-factor ANOVA was used to determine significant changes in plasma amino acid concentration (5 × 7; condition, time), whole-body leucine oxidation (5 × 6; condition, time), plasma urea concentration (2 × 5; time, condition), and protein phosphorylation (2 × 5; time, condition). Differences in means were determined using a Holm-Sidak post hoc test. All data were analyzed using SigmaStat 3.1 statistical software (Systat Software Inc, Chicago, IL), and statistical significance was set at $P \leq 0.05$. Values are expressed as mean ± SEM.

RESULTS

Blood amino acid concentrations are summarized in **Table 1**. There was no change in EAA, BCAA, or leucine concentrations after exercise in the absence of protein ingestion ($P > 0.05$). There was a slight increase ($P < 0.01$) in BCAA concentration 0.75–1 h after ingestion of 5 g protein that was not significantly different from 0 g (main effect for condition, $P > 0.05$). EAA, BCAA, and leucine concentrations were greater after ingestion of 10 g compared with 0 g (main effect for condition, $P < 0.01$) and peaked at 0.75 h after exercise ($P < 0.01$). After ingestion of 20 g protein, concentrations of EAA and BCAA peaked at 0.75 h ($P < 0.01$) and were >0 and 5 g (main effect for condition, $P < 0.01$), whereas leucine was only >0 g (main effect for condition, $P < 0.01$). Ingestion of 40 g protein increased EAA, BCAA, and leucine concentrations by 0.75 h that remained elevated for the duration of the trial ($P < 0.01$) and were greater than all other conditions (main effect for condition, $P < 0.01$).

Baseline plasma insulin concentrations (4.5 ± 0.7, 5.8 ± 0.7, 5.0 ± 0.7, 5.6 ± 0.5, and 6.1 ± 0.9 μU/mL, conditions 0–40 g, respectively; $P > 0.05$) were similar in all conditions. Although insulin concentrations were not significantly altered over time (time × condition interaction not significant, $P > 0.05$), the insulin area under the curve over 4 h with 40 g (1648 ± 202 μU/mL) protein was greater ($P < 0.01$) than after 0, 5, and 10 g (1040 ± 98, 1186 ± 82, and 1258 ± 201 μU/mL, respectively) and tended to be greater than after 20 g (1340 ± 118 μU/mL; $P = 0.009$, critical level = 0.007) protein ingestion. Baseline blood glucose concentration (4.9 ± 0.6, 4.5 ± 0.4, 4.7 ± 0.5, 5.0 ± 0.5, and 4.5 ± 0.5 mmol/L, conditions 0–40 g, respectively; $P > 0.05$) was similar for all conditions and remained steady throughout the entire study period (data not shown).

Plasma α-KIC enrichment was at plateau in all conditions (7.8 ± 0.2, 7.3 ± 0.2, 7.6 ± 0.1, 7.5 ± 0.3, and 8.3 ± 0.2 atom percent excess for conditions 0–40 g, respectively). Mixed-muscle

TABLE 1
Blood amino acid concentrations after exercise[1]

	Time (h)						
Protein	0	0.75	1	1.5	2	3	4
				μmol/L			
EAAs							
0 g*[2]	628 (16)	609 (24)	637 (43)	618 (46)	566 (36)	524 (24)	537 (31)
5 g†	649 (37)[a,b,3]	706 (28)[b,c]	703 (23)[b,c]	697 (39)[a,b]	649 (19)[a,b]	581 (31)[a,b]	555 (35)[a]
10 g†‡	670 (8)[a,b]	782 (25)[c]	764 (41)[c]	760 (50)[c]	690 (19)[b,c]	621 (23)[b]	568 (35)[a]
20 g‡	656 (47)[a]	883 (78)[b]	864 (82)[b,c]	752 (47)[c,d]	731 (30)[a,d]	667 (15)[a]	625 (36)[a]
40 gδ	660 (25)[a]	895 (40)[b]	892 (53)[b]	883 (71)[b]	878 (66)[b]	841 (45)[b]	788 (48)[b]
BCAAs							
0 g*	274 (11)	256 (12)	266 (21)	255 (23)	241 (17)	232 (10)	244 (13)
5 g*†	288 (22)	313 (18)	309 (15)	308 (19)	290 (13)	262 (17)	251 (18)
10 g†‡	298 (9)[a,b]	362 (19)[c]	346 (24)[b,c]	353 (32)[a–c]	323 (15)[a–c]	288 (12)[a]	272 (20)[a]
20 g‡	296 (22)[a]	420 (40)[c]	412 (44)[c]	360 (34)[b,c]	353 (23)[a–c]	326 (14)[a,b]	311 (15)[a,b]
40 gδ	303 (17)[a]	451 (31)[b]	454 (38)[b]	448 (46)[b]	456 (46)[b]	441 (34)[b]	413 (31)[b]
Leucine							
0 g*	82 (3)	83 (3)	86 (6)	88 (6)	83 (7)	84 (4)	90 (5)
5 g*†	86 (5)	101 (4)	100 (3)	101 (5)	97 (2)	90 (5)	91 (6)
10 g*†	88 (2)[a]	120 (6)[c]	112 (7)[b,c]	115 (8)[a–c]	106 (4)[a–c]	99 (4)[a–c]	96 (6)[a,b]
20 g†	88 (6)[a]	146 (15)[d]	136 (13)[c,d]	118 (9)[c]	116 (5)[b,c]	108 (3)[a–c]	106 (4)[a–c]
40 g‡	98 (10)[a]	167 (13)[c]	166 (12)[c]	155 (21)[b,c]	156 (18)[b,c]	148 (14)[b]	141 (13)[b]

[1] Values are means (SEM); $n = 6$. Blood amino acid concentrations were measured over 4 h after exercise. EAAs, essential amino acids (sum of His, Ile, Leu, Lys, Met, Phe, Thr, Val; note Cys not measured); BCAAs, branched-chain amino acids (sum of Ile, Leu, and Val). Data were analyzed using a 2-factor (protein × time) ANOVA. Differences in means were determined using a Holm-Sidak post hoc test. Protein × time interactions for EAAs, BCAAs, and leucine were all significant, $P < 0.01$.

[2] Protein doses with different symbols are significantly different from each other (main effect for protein, $P < 0.01$).

[3] Means within each protein dose with different superscript letters are significantly different from each other, $P < 0.01$.

FSR was increased ($P < 0.01$) above the fasted condition by ≈37 and 56% after consumption of 5 and 10 g protein, respectively (**Figure 2**). At 20 g protein, there was a ≈93% increase ($P < 0.01$) in mixed-muscle FSR above the fasted condition. There was no difference in mixed-muscle FSR after consumption of 20 or 40 g protein ($P = 0.29$). Similarly, plasma albumin FSR increased ($P < 0.01$) in a dose-dependent manner in response to increasing amounts of dietary protein and reached a plateau at 20 g (**Figure 3**). There was no difference in albumin FSR after consumption of 20 or 40 g dietary protein ($P = 0.65$). The average baseline plasma albumin concentration was 4.5 ± 0.4 g/dL with no differences between conditions (data not shown). A plot of the residuals for mixed muscle and plasma albumin protein synthesis revealed no bias of trial order on the measurement of FSR (data not shown).

Despite the marked changes in mixed-muscle FSR, there was no statistically significant change ($P > 0.05$) in the phosphorylation of S6K1 (Thr^{389}), rps6 ($Ser^{240/244}$), or eIF2Bε (Ser^{539}) 1 or 4 h after exercise with increasing protein intake (**Figure 4**).

Leucine oxidation was not different ($P > 0.05$) from the fasted condition after ingestion of 5 and 10 g protein (**Table 2**). Leucine oxidation was stimulated at both 20 and 40 g dietary protein (main effect for condition; $P > 0.05$). There was a trend toward a greater area under the leucine oxidation by time curve (estimate of total leucine oxidized over 4 h) after ingestion of 40 g compared with 20 g protein (153.1 ± 12.3 compared with 129.3 ± 9.0 μmol/kg; $P = 0.017$, critical level = 0.013). Plasma urea was similar at baseline for all conditions (3.6 ± 0.2, 3.5 ± 0.5, 3.7 ± 0.3, 3.7 ± 0.3, and 4.0 ± 0.1 mmol/L, conditions 0–40 g, respectively; $P > 0.05$). At 4 h, there were slight decreases in plasma urea after ingestion of 0 and 5 g (≈3 and ≈10%, respectively) and slight increases after ingestion of 10, 20, and 40 g protein (≈7, ≈5, and ≈7%, respectively), although none of these changes were significant ($P > 0.1$ for all comparisons).

DISCUSSION

Our study is the first to our knowledge to describe the responses of mixed-muscle and albumin protein synthesis as well as whole-body leucine oxidation to increasing protein intake after an acute bout of resistance exercise. We report that increasing protein intake stimulates mixed-muscle and plasma albumin protein synthesis in a dose-dependent manner up to 20 g dietary protein, after which there is a marked stimulation of whole-body leucine oxidation and no further increase in protein synthesis. The stimulation of muscle protein synthesis with increasing protein intake was not associated with any consistent change in the phosphorylation status of candidate signaling proteins.

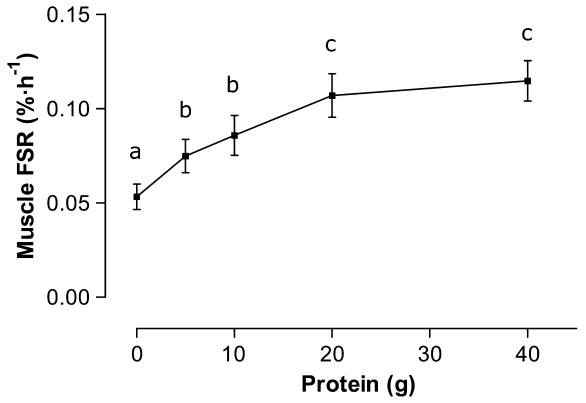


FIGURE 2. Mean (±SEM) mixed-muscle fractional protein synthesis (FSR) after resistance exercise in response to increasing amounts of dietary protein. Data were analyzed using a one-factor (protein) repeated-measures ANOVA to test for differences between conditions. Differences in means were analyzed using a Holm-Sidak post hoc test. Means with different letters are significantly different from each other ($P < 0.01$; $n = 6$).

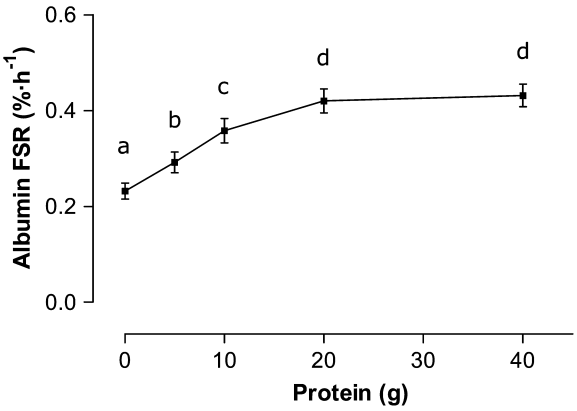


FIGURE 3. Mean (±SEM) plasma albumin fractional protein synthesis (FSR) after resistance exercise in response to increasing amounts of dietary protein. Data were analyzed using a one-factor (protein) repeated-measures ANOVA to test for differences between conditions. Differences in means were analyzed using a Holm-Sidak post hoc test. Means with different letters are significantly different from each other ($P < 0.01$; $n = 6$).

Borsheim et al (8) proposed the existence of a dose-response relation between muscle protein synthesis and amino acid consumption after resistance exercise on the basis of a comparison of data from 2 studies (8, 9). They observed a postexercise stimulation of muscle protein synthesis almost twice as great after ingestion of 6 g compared with only 3 g EAAs (8, 9). Our present findings are in agreement with their conclusion in that there is an apparent graded response of muscle protein synthesis to low doses of dietary protein, as suggested by the trend ($P = 0.059$, critical level = 0.025) toward a greater protein synthetic response after ingestion of 10 g (≈4.3 g EAAs) compared with 5 g (≈2.2 g EAAs) whole protein. This is similar to our previously published data demonstrating that 10 g whey protein is sufficient to enhance the postexercise stimulation of muscle protein synthesis (4). The current data expand on previous work that shows that muscle protein synthesis is further stimulated with greater protein intakes but reaches a plateau after ingestion of 20 g high-quality protein (≈8.6 g EAAs). This is in agreement with a previous observation showing similar postexercise net amino acid balance after 2 high doses of EAAs (≈21 compared with 40 g) (2). Thus, it appears that there is a maximal effective dose of dietary amino acids for stimulating muscle anabolism after resistance exercise. More important, our data suggest that the dose of EAAs that maximally stimulates muscle protein synthesis after resistance exercise (≈8.6 g) is very similar to that seen at rest (10 g) (7).

The phosphorylation of intracellular signaling proteins we measured was unaffected by protein ingestion. This is in contrast to previous work that has shown that amino acids robustly enhance mTOR signaling and the activity of S6K1 and eIF2B (7, 13, 19). However, all of our measurements of the phosphorylation state of

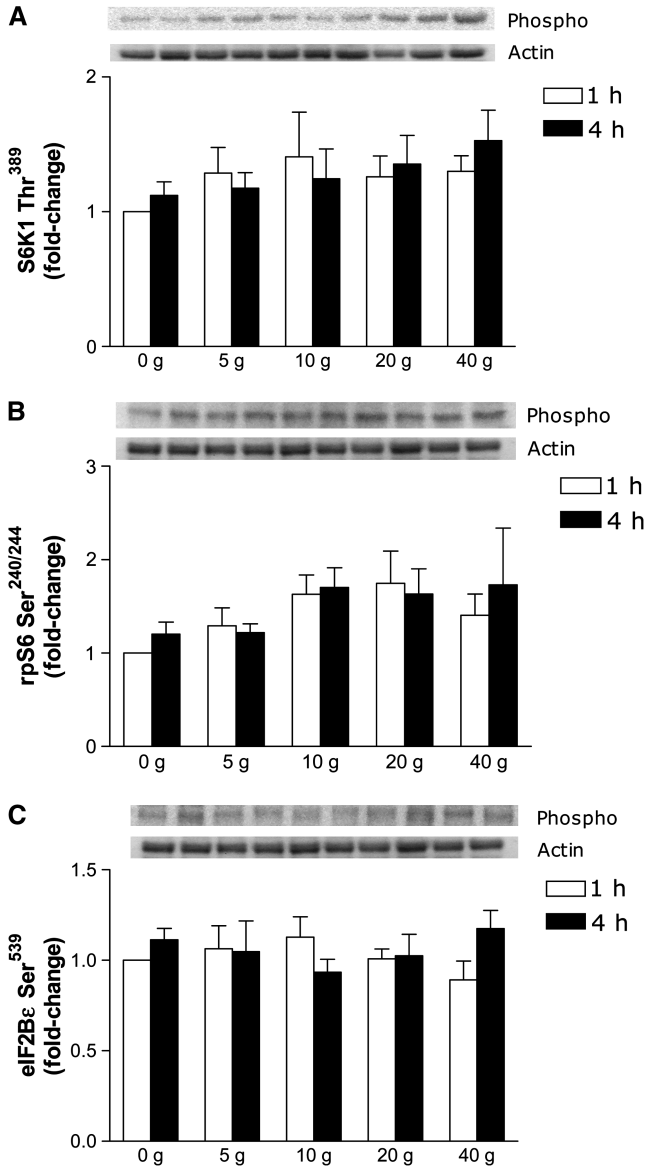


FIGURE 4. Mean (±SEM) phosphorylation of S6K1 at Thr^{389} (A), rpS6 at $Ser^{240/244}$ (B), and eIF2B at Ser^{539} (C) 1 and 4 h after exercise in response to increasing amounts of dietary protein. Data are expressed as a fold-change from 0 g protein ingestion at 1 h. A separate gel was run for each participant with 1- and 4-h samples loaded in adjacent lanes for all conditions (0–40 g protein, respectively). Gel orientation was identical for all proteins. Data were analyzed using a 2-factor (protein × time) repeated-measures ANOVA. There were no significant main effects for time or protein ($P > 0.05$). There was no significant time × protein interaction ($P > 0.05$; $n = 6$).

these proteins were made against a background of resistance exercise, which is a potent anabolic stimulus that has also been shown to increase the activity of these signaling pathways in the fasted state both in rats (18, 34) and humans (11, 12, 15, 16, 35). Thus, it is possible that resistance exercise already stimulated the phosphorylation of proteins within the mTOR-signaling pathway, thereby masking any amino acid–induced changes with protein ingestion in our representative downstream effectors. Although this would seem at odds with work demonstrating the phosphorylation of S6K1 is enhanced with feeding after exercise (12, 14, 16, 36), these studies provided either additional carbohydrate, which would stimulate insulin release that enhances the activation

TABLE 2
Whole-body leucine oxidation after exercise[1]

	Time (h)					
Protein	1[a,2]	1.5[a,b]	2[b,c]	2.5[b,c]	3[c,d]	4[d]
	$\mu mol \cdot kg^{-1} \cdot h^{-1}$					
0 g*[,3]	34 (4)	25 (2)	22 (2)	21 (2)	21 (3)	20 (3)
5 g*	37 (2)	32 (1)	31 (2)	27 (2)	27 (3)	23 (2)
10 g*	36 (3)	36 (5)	30 (3)	29 (3)	27 (3)	25 (2)
20 g†	48 (4)	49 (3)	45 (4)	44 (3)	40 (3)	36 (2)
40 g†	49 (4)	55 (4)	57 (4)	56 (7)	48 (6)	41 (4)

[1] Values are means (SEM); $n = 6$. Whole-body leucine oxidation was measured over 4 h after exercise. Data were analyzed using a 2-factor (protein × time) ANOVA. Differences in means were determined using a Holm-Sidak post hoc test.

[2] Times with different superscript letters are significantly different (main effect for time, $P < 0.01$). There was no significant interaction (protein × time, $P > 0.05$).

[3] Protein doses with different symbols are significantly different from each other (main effect for protein, $P < 0.01$).

of the mTOR-signaling cascade in the presence of amino acids (37, 38), or a source of crystalline EAA before, during, and after exercise and are therefore difficult to compare with the present study in which postexercise feeding of intact proteins was used. Alternatively, training status has also been shown to influence signal transduction after exercise, and it is possible that the resistance-trained background of the individuals in the present study may have resulted in a relatively blunted signaling response to exercise and feeding (39). Moreover, although a reduction in phosphorylation at residue Ser^{539} releases eIF2Bε from the inhibitory state mediated by GSK3β and likely contributes to an increase in its guanine nucleotide exchange activity (40), Wang and Proud (41) recently demonstrated that amino acids repress the phosphorylation of eIF2Bε on an as-yet-unstudied (in humans) residue (Ser^{525}), which may be the main mechanism by which amino acids regulate eIF2B activity; this clearly warrants further investigation. Nonetheless, our data demonstrate that the phosphorylation status of certain signaling proteins, at least at the time points we studied, shed little light on what is determining the rate of muscle protein synthesis with protein ingestion after exercise.

Because we saw no consistent change in the phosphorylation of candidate signaling proteins, a reasonable question is what is regulating muscle protein synthesis after resistance exercise with increasing protein ingestion. We speculate that an increase in delivery of amino acids might be the stimulus—that is, the postexercise stimulation of muscle protein synthesis is primarily related to substrate (ie, amino acid) availability (1). In short, resistance exercise as an anabolic stimulus would facilitate the transport of amino acids into the muscle (1, 42) and would also prime the translational machinery of the muscle cell [*see* (10) for review]. Amino acids taken up from the circulation are then rapidly incorporated into new tissue proteins. Our data show that there is, however, a maximal rate at which dietary amino acids can be incorporated into muscle tissue and that with increasingly higher concentrations of amino acids, there is no further stimulation of muscle protein synthesis.

We observed a dose-dependent stimulation of APS to increasing amounts of dietary protein. Albumin synthesis appears unaffected by resistance exercise (20), suggesting that the

differences in its fractional synthetic rate we observed is amino acid mediated. This is consistent with the observation that albumin protein synthesis is increased in response to the consumption of a protein-containing meal (21–23) and can be influenced by the level of protein in the diet (23). In fact, one thesis is that dietary amino acids consumed in excess of their acute requirement to synthesize lean tissue are directed toward albumin synthesis to minimize their irreversible oxidative loss (21). In the present study, rates of albumin synthesis (eg, mixed muscle proteins) reached a plateau at 20 g ingested protein, after which there was a marked stimulation of whole-body leucine oxidation. Consequently, whereas a fraction of dietary amino acids may be sequestered in albumin protein as a conservatory mechanism, we speculate that this is relatively minor in comparison with the large storage capacity of skeletal muscle. It is more likely that, similar to other lean tissues such as the splanchnic region (43), feeding stimulates turnover (ie, synthesis and degradation) of plasma albumin with little expansion of the protein pool.

With graded protein intakes, the point at which amino acid oxidation significantly increases has been suggested to reflect the level at which protein intake becomes excessive (44). Suggestive of a nutrient excess (45), leucine oxidation in the present study was stimulated after ingestion of 20 and 40 g protein. In addition, muscle and plasma albumin protein synthesis were maximally stimulated at 20 g dietary protein, which suggests an upper limit for incorporation of amino into these protein pools had been reached.

Provided that adequate energy intake is met (46), our findings have implications for protein recommendations for resistance-trained athletes in terms of the quantity of dietary protein that might maximize muscle growth. If we assume that a 20-g protein dose maximally stimulates muscle protein synthesis after exercise and we know that resistance exercise enhances the synthesis of muscle protein for at least 24 h (26, 47, 48), one could ask how many times in a day could someone consume such a dose to stimulate muscle anabolism that would ultimately translate into muscle growth? Because muscle protein synthesis becomes refractory to persistent aminoacidemia (49) and excess amino acids are lost to oxidation (44), we speculate that no more than 5–6 times daily could one ingest this amount ($\approx$20 g) of protein and expect muscle protein synthesis to be maximally stimulated. Protein consumption in excess of this rate or dose would ultimately lead to oxidative loss. In addition, given that the capacity to oxidize amino acids adapts to the diet and can act as a key regulator of protein stores (50), chronic protein consumption in excess of this rate or dose could actually lead to dampening of the protein synthetic response to suboptimal (ie, $<$20 g) protein doses.

In summary, our data are the first, to our knowledge, to demonstrate that muscle protein synthesis responds to increasing protein intake in a dose-dependent manner after resistance exercise and reaches a maximal stimulation after ingestion of 20 g high-quality protein. We observed little change in the phosphorylation status of signaling proteins shown to activate the translational machinery, which suggests that the main driver of muscle anabolism with feeding after resistance exercise may simply be amino acid availability. We also report that APS is stimulated by increasing protein ingestion after resistance exercise and reached a plateau at 20 g ingested protein. The stimulation of leucine oxidation after ingestion of 20 and 40 g protein suggests that these doses represent an excess of protein. In conclusion, our data indicate that 20 g intact high-quality protein is sufficient to maximize the anabolic response to resistance exercise.

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The authors' responsibilities were as follows—DRM, JET, MAT, and SMP: planned the study; DRM, MJR, JLF, TP, and SMP: collected the data; DRM, MJR, JLF, JET, EIG, SBW, and TP: analyzed the data; and DRM, JET, EIG, MAT, and SMP: wrote and edited the manuscript. None of the authors had any financial or personal conflicts of interest to declare.

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