

Muscle full effect after oral protein: time-dependent concordance and discordance between human muscle protein synthesis and mTORC1 signaling^{1–4}

Philip J Atherton, Timothy Etheridge, Peter W Watt, Daniel Wilkinson, Anna Selby, Debbie Rankin, Ken Smith, and Michael J Rennie

ABSTRACT

Background: We previously showed that human muscle protein synthesis (MPS) increased during infusion of amino acids (AAs) and peaked at ≈ 120 min before returning to baseline rates, despite elevated plasma AA concentrations.

Objective: We tested whether a protein meal elicited a similar response and whether signaling responses that regulate messenger RNA translation matched MPS changes.

Design: Eight postabsorptive healthy men (≈ 21 y of age) were studied during 8.5 h of primed continuous infusion of [1,2-¹³C₂] leucine with intermittent quadriceps biopsies for determination of MPS and anabolic signaling. After 2.5 h, subjects consumed 48 g whey protein.

Results: At 45–90 min after oral protein bolus, mean (\pm SEM) myofibrillar protein synthesis increased from $0.03 \pm 0.003\%$ to $0.10 \pm 0.01\%/h$; thereafter, myofibrillar protein synthesis returned to baseline rates even though plasma essential AA (EAA) concentrations remained elevated ($+130\%$ at 120 min, $+80\%$ at 180 min). The activity of protein kinase B (PKB) and phosphorylation of eukaryotic initiation factor 4G preceded the rise of MPS and increases in phosphorylation of ribosomal protein kinase S6 (S6K1), and 4E-binding protein 1 (4EBP1) was superimposable with MPS responses until 90 min. However, although MPS decreased thereafter, all signals, with the exception of PKB activity (which mirrored insulin responses), remained elevated, which echoed the slowly declining plasma EAA profile. The phosphorylation of eukaryotic initiation factor 2 α increased only at 180 min. Thus, discordance existed between MPS and the mammalian target of rapamycin complex 1 (mTORC1) and signaling (ie, S6K1 and 4EBP1 phosphorylation).

Conclusions: We confirm our previous findings that MPS responses to AAs are transient, even with oral protein bolus. However, changes in MPS only reflect elevated mTORC1 signaling during the upswing in MPS. *Am J Clin Nutr* 2010;92:1080–8.

INTRODUCTION

The first demonstration that a mixed meal was able to stimulate human muscle protein synthesis (MPS) was made in 1982 (1), and 7 y later it was shown that amino acids (AAs) alone were likely to make the major contribution to the stimulation (2). When these early measurements were made, technical limitations made it difficult to measure MPS over short periods so that most of the reported measurements were made over ≥ 6 –8 h (3). However,

improvements in analytic sensitivity led one of the authors (MJR), with other workers, to investigate the latency and duration of MPS responses to increased AA availability. We showed that, in response to constant intravenous infusion of AAs, there was a latent period before MPS approximately trebled between 30–120 min (4), thereafter returning to postabsorptive rates, despite the continued elevated availability of AAs for up to a further 4 h. These findings were linked to the “muscle full” hypothesis (5), in which it was suggested that there must be an upper limit of AA delivery before muscle cells would no longer use them as a substrate for MPS, instead diverting them toward oxidation (4). In a further demonstration of the relation between AA availability and MPS, Rennie, again with other workers, established that responses of MPS are related to the blood concentration of AAs, especially of essential AAs (EAAs) (6) and, in particular, leucine, in a dose-dependent and saturable fashion; later, it was shown that a similar relation exists between MPS and the amount of orally administered EAAs, which was maximal at ≈ 20 g EAAs (7). Other workers showed a similar relation for protein (8).

Intriguingly, increased AA availability provides substrate for protein synthesis and stimulates intramuscular signaling events that regulate the initiation and elongation phases of messenger RNA (mRNA) translation. Of the signals that regulate MPS, a large body of work that reported intramuscular signaling concurrent with measures of MPS in rodent (9, 10) and human (7,

¹ From the School of Graduate Entry Medicine and Health, Division of Clinical Physiology, University of Nottingham, Royal Derby Hospital, Derby, United Kingdom (PJA, TE, AS, DR, KS, and MJR), and the Chelsea School, Hillbrow, University of Brighton, Eastbourne, United Kingdom (TE, PWW, and DW).

² PJA and TE contributed equally to this study.

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⁴ Address correspondence to PJ Atherton or MJ Rennie, School of Graduate Entry Medicine and Health, Division of Clinical Physiology, University of Nottingham, Royal Derby Hospital, Uttoxeter Road, Derby, DE22 3DT, United Kingdom. E-mail: philip.atherton@nottingham.ac.uk or michael.rennie@nottingham.ac.uk.

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11, 12) muscles indicated a central role for the mammalian target of rapamycin complex 1 (mTORC1) pathway. Activation of mTORC1 is linked to phosphorylation of ribosomal protein kinase S6 (S6K1) (13) and 4E-binding protein 1 (4EBP1) (14), among many other substrates (15). Proximal AA sensing is poorly defined but may be linked to mitogen-activated protein kinase kinase kinase 3 (16), vacuolar protein sorting 34, (17) and the recombination activating G proteins (Rags) (18).

To our knowledge, no researchers have established the time courses of responses of human MPS to a single meal of protein of a nutritionally meaningful size or the signaling responses thought to be involved in the control of mRNA translation (9, 12). We aimed to fill this gap. We hypothesized that 1) a protein meal (48 g whey) would, like AA infusion, also stimulate a short-lived response, despite causing longer, more sustained elevations in plasma and muscle intramuscular AAs, beyond the peak MPS response, and 2) molecular signaling responses that regulate mRNA translation would prefigure the molecular signaling responses of MPS.

SUBJECTS AND METHODS

Study design

Ethical approval was obtained from the University of Nottingham committee (United Kingdom), with all studies being carried out in accordance with the Declaration of Helsinki. Recruitment for healthy young men [$n = 8$; 21 ± 2 y old (mean \pm SEM); body mass index (in kg/m^2): 22.9 ± 0.9] began in January 2008. All recruits (who were recreationally active but involved in no formal training program) were studied after an overnight fast and were asked to refrain from heavy exercise for 72 h before the study day. On the morning of the study (≈ 0800), subjects had 18-g cannulae inserted into the antecubital veins of both arms for tracer infusion and blood sampling. Blood samples and muscle biopsies were taken according to the protocol (Figure 1). Muscle biopsies were taken intermittently from the quadriceps under sterile conditions by using the conchotome technique (19) with 1% lignocaine as a local anesthetic. The muscle tissue was washed in ice-cold saline before visible fat and connective tissue were removed, and it was immediately frozen in liquid nitrogen and stored at -80°C until further analysis. A primed continuous infusion ($0.7 \text{ mg}/\text{kg}$, $1 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$) of $[1, 2\text{-}^{13}\text{C}_2]\text{leucine}$ tracer (99 Atoms %; Cambridge Isotopes Limited, Cambridge, MA) was started (at 0 h) after the first biopsy and maintained until the end of the study (at 8.5 h). A second biopsy was taken after 2.5 h in the postabsorptive state

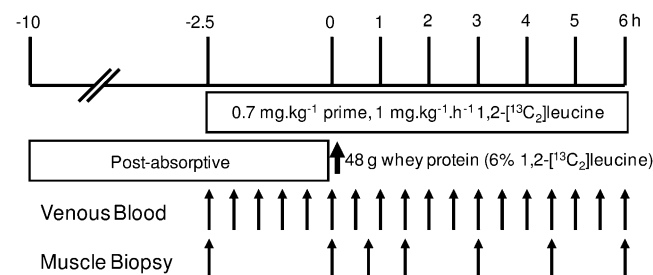


FIGURE 1. Protocol for the measurement of muscle protein synthesis and anabolic signaling phosphorylation in response to ingestion of 48 g whey protein.

so that the basal rate of MPS could be measured, whereupon the subjects drank a preparation of 48 g whey-protein isolate (98% whey, 1% CHO, and 1% fat; $\approx 840 \text{ kJ}$) powder in 500 mL water (equivalent to $\approx 20 \text{ g EAA}$). After the study, both catheters were removed, and subjects were fed and monitored for 30 min before being allowed to go home.

Measurement of plasma AA, glucose, and insulin concentrations

Plasma glucose concentrations were measured with a Clinical Chemistry System (iLab 300 plus; Instrumentation Laboratory, Cheshire, United Kingdom). Plasma insulin concentrations were measured by using a human high sensitivity enzyme-linked immunosorbent assay (DRG Instruments GmbH, Marburg, Germany). For AA analyses, equal volumes of plasma and 10% sulfosalicylic acid were mixed and cooled at 4°C for 30 min. Samples were centrifuged at $8000 \times g$ to pellet the precipitated

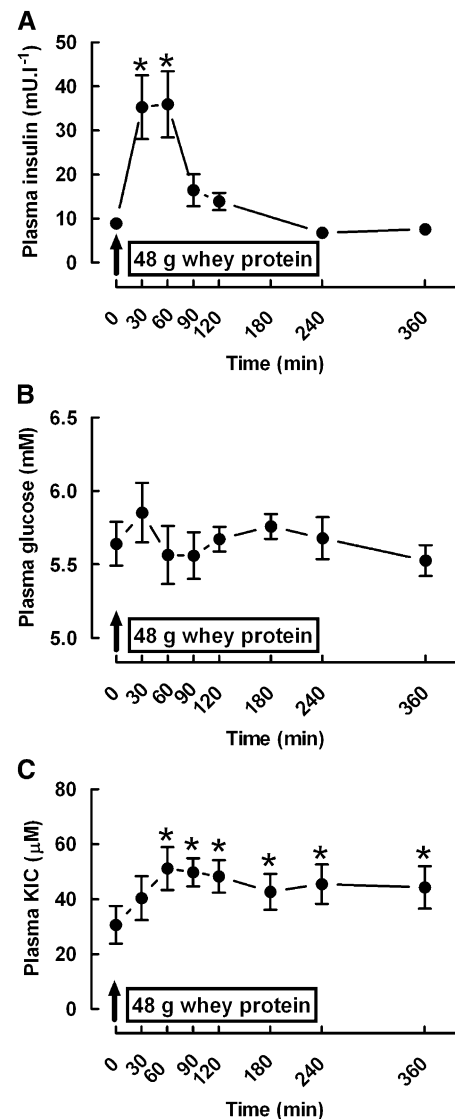


FIGURE 2. Mean (\pm SEM) concentrations of plasma insulin (A), glucose (B), and α -ketoisocaproate (KIC) (C) in response to a 48-g whey-protein bolus ($n = 8$). *Significant increase from postabsorptive values, $P < 0.01$ (repeated-measures ANOVA with Tukey's post hoc test).

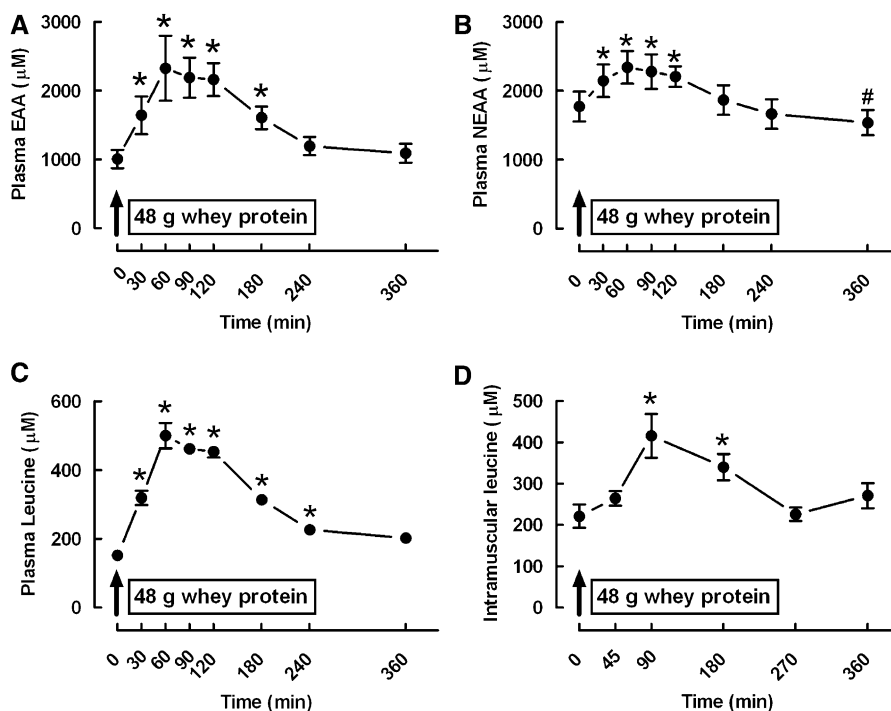


FIGURE 3. Mean (\pm SEM) concentrations of plasma essential amino acid (EAA) (A), nonessential AA (NEAA) (B), leucine (C), and intramuscular concentrations of leucine (D) in response to a 48-g whey-protein bolus ($n = 8$). *Significant increase from postabsorptive values, $P < 0.05$ (repeated-measures ANOVA with Tukey's post hoc test). #Significant decrease from postabsorptive values, $P < 0.05$ (repeated measures ANOVA with Tukey's post hoc test).

protein, and the supernatant fluid was passed through a 0.22- μ m filter before analysis with dedicated AA analyzer (Biochrom 30; Biochrom, Cambridge, United Kingdom) by using lithium buffers. All 20-AA concentrations were measured by comparison to a standard AA mix by using norleucine as an internal standard.

Muscle preparation for tracer leucine incorporation and intramuscular AA analyses

Briefly, muscle tissue (≈ 25 mg) was minced with scissors in ice-cold extraction buffer that contained 50 mmol tris HCl/L (pH 7.4), 1 mmol EGTA/L, 1 mmol EDTA/L, 10 mmol β -glycerophosphate/L, 50 mmol NaF/L, 0.5 mmol activated sodium orthovanadate/L (all from Sigma-Aldrich, Poole, United Kingdom), and a complete protease inhibitor cocktail tablet (Roche, West Sussex, United Kingdom). The resulting homogenate was centrifuged at $13,000 \times g$ for 20 min to pellet the myofibrillar fraction. The supernatant fluid was used for determination of the sarcoplasmic protein fractional synthetic rate (FSR) via precipitating out protein by using 1 mol perchloric acid/L and washing twice with 70% ethanol. The myofibrillar pellet was solubilized with 0.3 mol NaOH/L and centrifuged at $3000 \times g$ for 20 min to separate it from the insoluble collagen fraction. The solubilized myofibrillar protein was precipitated by using ice-cold 1 mol perchloric acid/L, the resulting pellet was washed twice with 70% ethanol and collected by centrifugation. Protein-bound AAs were released by acid hydrolysis in a Dowex H^+ resin slurry (0.05 mol HCl/L; Sigma-Aldrich, Poole, United Kingdom) at 110°C overnight. AAs were purified by ion-exchange chromatography on Dowex H^+ resin. AAs were derivatized as their *N*-acetyl-*N*-propyl esters as previously de-

scribed (20). Incorporation of $[1, 2-^{13}\text{C}_2]$ leucine into protein was measured by gas chromatography–combustion-isotope ratio mass spectrometry (Delta plus XP; Thermofisher Scientific, Hemel Hempstead, United Kingdom) by using our standard techniques (21). Intramuscular leucine concentrations were measured after precipitating the sarcoplasmic fraction with ethanol, before drying the resulting supernatant fluid and taking up the lithium buffer. Concentrations of leucine were calculated as described (22). Venous plasma α -ketoisocaproate was chosen as the surrogate precursor for leucyl-*t*-RNA labeling; briefly, plasma protein was precipitated and the quinoxalinol derivative was formed and extracted into ethyl acetate, dried down, and derivatized as its *t*-butyldimethylsilyl-quinoxalinol. Labelings and concentrations were measured by gas chromatography–mass spectrometry, α -ketovaleate was used as the internal standard, and measurements were performed relative to a standard curve.

Myofibrillar and sarcoplasmic protein synthetic rates

The FSR of the protein was measured from the increase in incorporation of $[1, 2-^{13}\text{C}_2]$ leucine between subsequent muscle biopsies by using the labeling of plasma α -ketoisocaproate as a surrogate of leucyl-*t*-RNA the precursor, as previously described (21). The FSR was calculated by using the standard precursor-product method as follows:

$$\text{Fractional protein synthesis (ks, \% / h)} = \frac{\text{Lm} / \text{Lp}}{\times 1 \div t \times 100} \quad (I)$$

where ks is the rate constant for synthesis, Lm is the change in leucine labeling between 2 biopsy samples, Lp is the mean

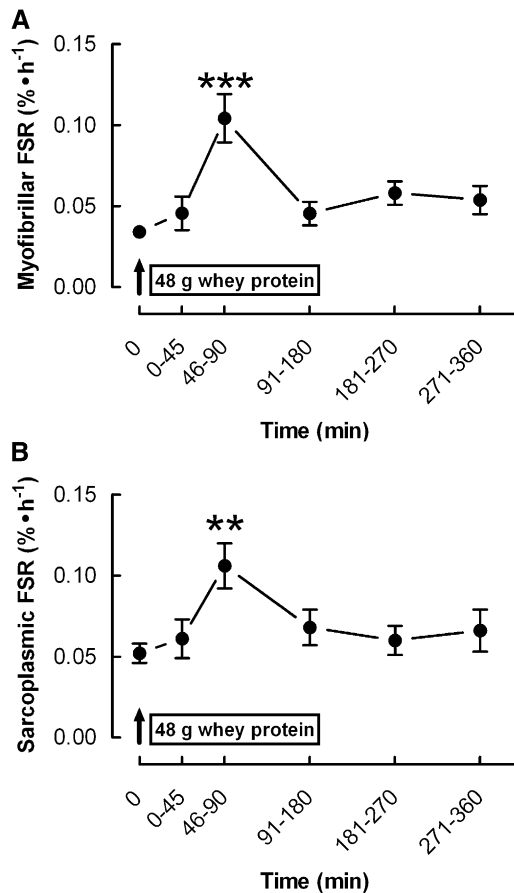


FIGURE 4. Mean (\pm SEM) myofibrillar (A) and sarcoplasmic (B) fractional synthetic rates (FSRs) in response to a 48-g whey-protein bolus ($n = 8$). *****Significant increase from postabsorptive values (repeated-measures ANOVA with Tukey's post hoc test): ** $P < 0.01$, *** $P < 0.0001$.

labeling over time of the precursor (ie, venous α -ketoisocaproate labeling), and t is the time between biopsies in hours.

Immunoblotting and protein kinase B kinase assay

Proteins were extracted from muscle (≈ 20 mg) as previously described (23). For kinase assays, protein kinase B (PKB) was immunoprecipitated from 200 μ g protein before incubation with 1 mmol ATP/L and glycogen synthase kinase 3 β (GSK3 β) serine (Ser) 9 fusion protein substrate (New England Biolabs, Hertfordshire, United Kingdom) before immunoblotting for GSK3 β Ser9 (15 μ L kinase assay) (24). For other phosphoproteins, samples were standardized to 1 mg/mL by dilution with 3 \times Laemmli loading buffer to measure relative phosphorylated protein concentrations of mammalian target of rapamycin (mTOR) Ser2448, S6K1 threonine (Thr) 389, 4EBP1 Thr37/46, eukaryotic initiation factor (eIF)-4E Ser209, extracellular regulated kinase (ERK)-1/2 Tyr202/204, eukaryotic elongation factor (eEF)-2 Thr56, eIF4G Ser1108, AMP-activated protein kinase (AMPK) Thr172, eIF2B ϵ Ser535, eIF2 α Ser51, and pan-actin as a loading control (Sigma Aldrich, Poole, United Kingdom, and New England Biolabs; all 1:2000). Fifteen micrograms of protein per lane was loaded on to 26-lane 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis gels (Criterion XT

Bis-Tris; Bio-Rad, Hemel Hempstead, United Kingdom) for immunoblotting, which was performed as described (23).

Interactions of components of the eIF4F complex

Total eIF4E was either immunoprecipitated by using 2.5 μ g of monoclonal antibody (New England Biolabs) or affinity purified by using 30 μ L 7-methyl conjugated GTP coupled sepharose slurry (GE Health Care Sciences, Buckinghamshire, United Kingdom) from 300 μ g sarcoplasmic protein overnight at 4°C with continuous rotation. Immunoprecipitates were captured by using protein-G coupled sepharose for 2 h at 4°C (Sigma-Aldrich), and beads were washed for both assays after pulse centrifugation in homogenization buffer. Bound proteins were eluted with 1 \times Laemmli buffer, boiled for 7 min, and blotted for total 4EBP1, eIF4G, and eIF4E (New England Biolabs).

Statistical analyses

Phosphorylation data were normalized to pan-actin to correct for loading anomalies and GSK3 β phosphorylation from the PKB kinase assays according to the total PKB recovery from immunoprecipitates. Binding of eIF4E to eIF4G and 4EBP1 was normalized to eIF4E recovery. Differences for all analyses were detected by repeated measures one-factor analysis of variance by using Tukey's post-hoc test with GraphPad software (version 5; GraphPad Software Inc, San Diego, CA), and $P < 0.05$ was considered significant. For temporal comparisons between FSR and other measures (eg, signaling protein phosphorylation), data sets were normalized over a range of 0–100% according to the data span (ie, for each data set, 0% represented the lowest value, and 100% represented the highest value). Data are presented as means \pm SEM.

RESULTS

Oral protein bolus-induced changes in plasma concentrations of insulin and glucose

After oral protein bolus, plasma insulin concentrations increased sharply and were elevated at 30 and 60 min (+296% and +303%, respectively; $P < 0.01$); however, by 90 min, plasma insulin concentrations were no different from postabsorptive values and remained so for the rest of the study. Plasma glucose concentrations were steady at typical postabsorptive values (≈ 5.5 mmol/L) throughout the study (Figure 2B). Plasma α -ketoisocaproate concentrations were increased by 60 min (+70%; $P < 0.01$) and remained elevated throughout the study (ie, 6 h after oral protein bolus; Figure 2C).

Oral protein bolus-induced changes in plasma concentrations of AA and intramuscular concentrations of leucine

Plasma EAA concentrations were significantly increased after 30 min, peaked at 60 min (+131%; $P < 0.01$), and remaining elevated for 180 min (Figure 3A), whereas nonessential AA concentrations, despite increasing at 30 min (+31%; $P = 0.05$), returned to basal values by 120 min, and by 360 min, non-essential AA concentrations were significantly depressed compared with postabsorptive concentrations (Figure 3B). Plasma

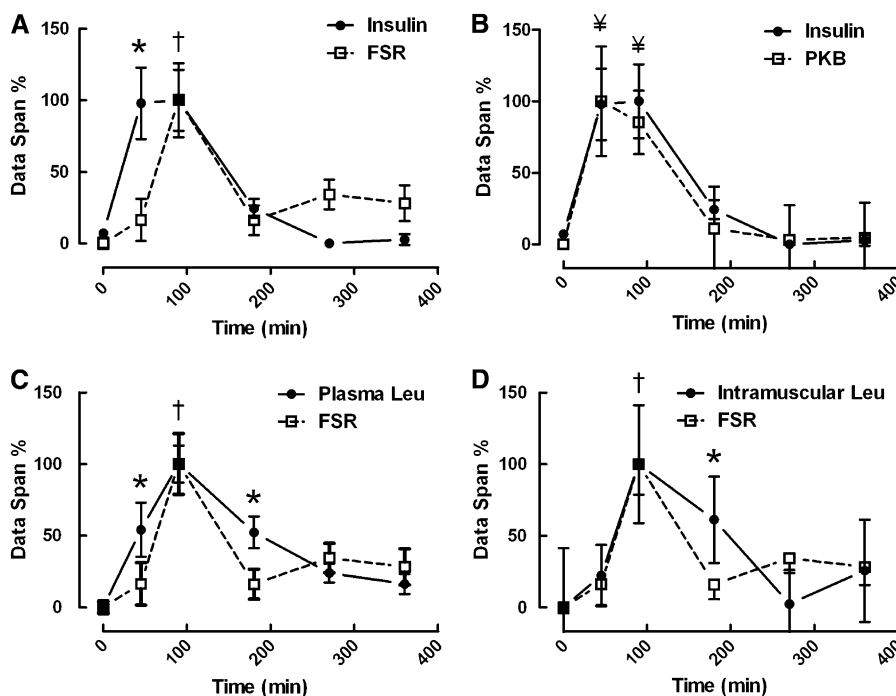


FIGURE 5. Mean (\pm SEM) comparisons between normalized (data span = 100%) responses of the fractional synthetic rate (FSR) and insulin with plasma and intramuscular leucine (Leu) and protein kinase B (PKB) activity. Myofibrillar FSR plotted with plasma insulin (A), plasma insulin plotted with PKB (B), and myofibrillar FSR plotted with plasma (C) and intramuscular (D) leucine in response to a single 48-g whey-protein bolus are shown ($n = 8$). *Significant increase from postabsorptive values in plasma or intramuscular leucine or insulin, $P < 0.05$ (repeated-measures ANOVA with Tukey's post hoc test). †Significant increase in FSR and plasma insulin, leucine, or intramuscular leucine, $P < 0.05$ (repeated-measures ANOVA with Tukey's post hoc test). ‡Significant increase in PKB kinase activity and plasma insulin, $P < 0.05$ (repeated-measures ANOVA with Tukey's post hoc test).

leucine concentrations were increased by 30 min, peaked at 60 min ($+129\%$; $P < 0.01$), and remained elevated until 240 min (Figure 3C), whereas intramuscular leucine concentrations (Figure 3D) increased for ≤ 180 min and peaked at 90 min ($+88\%$; $P < 0.01$); both leucine pools decreased to postabsorptive values thereafter.

Temporal response of myofibrillar and sarcoplasmic FSR to oral protein bolus

Neither myofibrillar nor sarcoplasmic FSR was altered 0–45 min after oral protein bolus. The myofibrillar FSR increased significantly by 46–90 min postfeed (0.034 ± 0.003 compared with $0.104 \pm 0.015\%/h$; $P < 0.05$) and returned to within baseline rates by 180 min, where it remained for the duration of the study (Figure 4). Sarcoplasmic FSR showed an identical pattern and increased by 46–90 min after oral protein bolus (0.052 ± 0.006 compared with $0.106 \pm 0.012\%/h$; $P < 0.05$) before returning to baseline values.

Comparison of normalized oral protein bolus-induced plasma insulin, leucine, and intramuscular leucine concentrations with myofibrillar FSR

When normalized for the data span, insulin concentration rises preceded FSR responses and fell with a similar pattern that was mirrored by PKB kinase activity (Figure 5, A and B; blots for PKB are shown in Figure 6A). In contrast, increases in plasma leucine concentrations preceded FSR responses but remained significantly elevated beyond the point at which the FSR had returned to postabsorptive values (Figure 5C). Similarly, in-

tramuscular leucine concentrations increased by 90 min and remained high even when the FSR had fallen back to postabsorptive values (Figure 5D).

Comparison of normalized oral protein bolus-induced changes in PKB activity and in S6K1, 4EBP1, and eIF4G phosphorylation with myofibrillar FSR

After the protein feed, the PKB activity increased rapidly ($+60\%$ and 50% at 45 and 90 min after oral protein bolus, respectively) before returning to postabsorptive values at later time points. The time course of the fold changes of PKB mirrored those of the myofibrillar FSR (Figure 6A). S6K1 phosphorylation mirrored that of myofibrillar protein synthesis initially and increased at 90 min ($+112\%$), but unlike FSR, S6K1 phosphorylation remained elevated at 180 min ($+50\%$) after oral protein bolus before falling toward postabsorptive values at 270 and 360 min (Figure 6B). However, the fall was slower than that of myofibrillar protein synthesis and no longer matched the changes in synthesis seen during their rise. 4EBP1 and eIF4G phosphorylation followed time courses similar to that of S6K1 and increased by $+160\%$ and $+120\%$ at 90 and 180 min, respectively (Figure 6, C and D).

Assembly of eIF4F complex components and eIF2 α

After the protein feed, the binding of eIF4E-4EBP1 was decreased at 90 min (-35%) and remained suppressed at 180 min (-23%) (Figure 7A). Conversely, the binding of eIF4E-eIF4G increased at 90 min ($+51\%$) and remained elevated at 180 min ($+29\%$) (Figure 7B). Binding was assessed by using immunoprecipitations in which the recovery of eIF4E was stable,

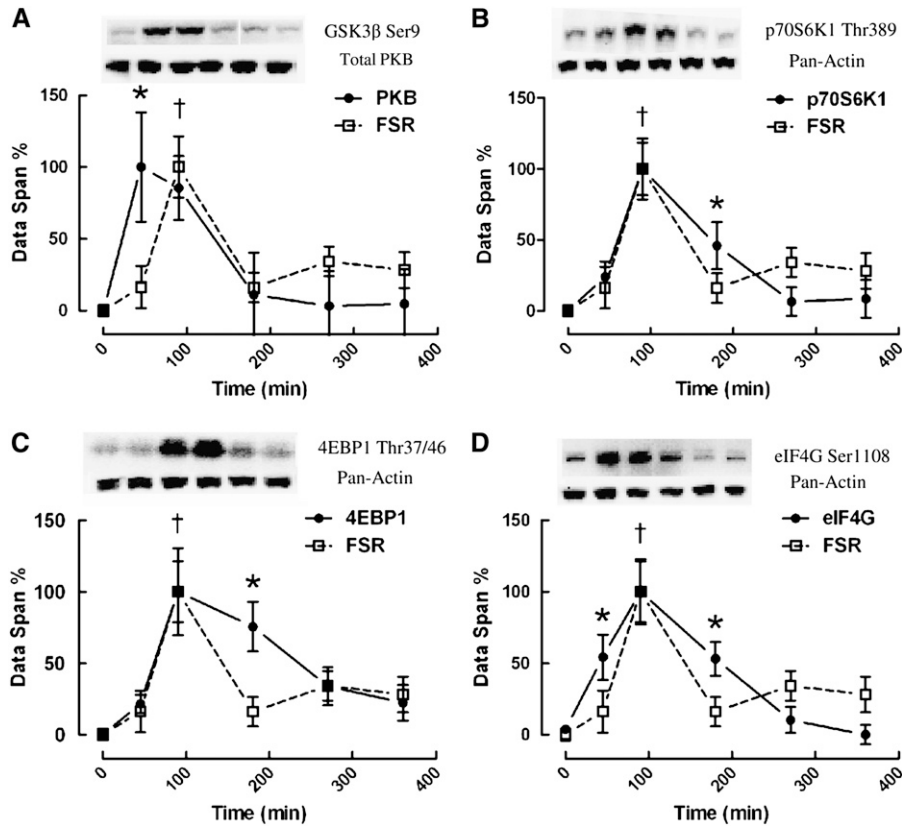


FIGURE 6. Mean (\pm SEM) comparisons between normalized (data span = 100%) responses of the fractional synthetic rate (FSR) with signals regulating messenger RNA translation. Myofibrillar FSRs plotted against protein kinase B (PKB) (A), ribosomal protein kinase S6 (p70S6K1) (B), 4E-binding protein 1 (4EBP1) (C), and eukaryotic initiation factor 4G (eIF4G) (D) in response to a single 48-g whey-protein bolus are shown ($n = 8$). *Significant increase from postabsorptive values in signaling protein phosphorylation, $P < 0.05$ (repeated-measures ANOVA with Tukey's post hoc test). †Significant increase in FSR and signaling protein phosphorylation, $P < 0.05$ (repeated-measures ANOVA with Tukey's post hoc test). GSK3 β , glycogen synthase kinase 3 β ; Ser, serine; Thr, threonine.

whereas the recovery of eIF4E was significantly lower at 90 and 180 min (-18%) when using an affinity purification with 7-methyl conjugated GTP sepharose (Figure 7C). There was a trend ($P = 0.07$) for an increase in eIF2 α phosphorylation ($+46\%$) by 180 min (Figure 7D).

Specific oral protein bolus-induced responses of AMPK, eEF2, ERK1/2, mTOR, and eIF4E phosphorylation

After ingestion of the protein meal, phosphorylation of AMPK and eEF2 remained within postabsorptive values throughout the study; the phosphorylation of ERK1/2, eIF4E, mTOR, and eIF2B ϵ were also unchanged (data not shown).

DISCUSSION

To our knowledge, we report in this study novel findings that represent the time courses in human beings of responses in myofibrillar protein synthesis (and its sarcoplasmic counterpart) and associated anabolic signaling to a physiologically relevant protein meal. In response to a single oral bolus of whey protein, we showed the following results: 1) after a latent period of ≈ 45 min, myofibrillar protein synthesis approximately tripled between 45 and 90 min before rapidly returning to postabsorptive rates, despite the continued availability of intramuscular leucine and plasma leucine and EAAs; 2) the increase of myofibrillar protein synthesis was preceded by the stimulation of PKB kinase ac-

tivity and phosphorylation of eIF4G and coincided with S6K1 and 4EBP1 phosphorylation; however, 3) contrary to our hypothesis, the rapid return to basal values in both myofibrillar and sarcoplasmic protein synthesis markedly preceded the dephosphorylation of S6K1, 4EBP1, and eIF4G in addition to eIF4F complex disassembly. The current results, together with those reported earlier (6), demonstrated that, in response to a saturable quantity of AAs, after a latent period of ≈ 30 – 45 min, rates of myofibrillar and sarcoplasmic protein synthesis were increased for a period of ≈ 60 – 90 min before rapidly returning to basal rates (4), irrespective of the mode of AA delivery (ie, oral protein compared with intravenous free AAs). Moreover, because MPS declined in the face of the continued availability of the activating stimulus (ie, plasma and intramuscular leucine), it is likely that muscle possesses a mechanism to gauge its capacity to synthesize new proteins. This would lead to the prediction that if kinases that regulate mRNA translation are sensitive to AAs per se, then such signals should mirror those of AA availability. In support of this, when we compared intramuscular leucine and plasma EAAs to mTOR substrates S6K1 and 4EBP1, an echoing decline was observed (ie, both returned to baseline concentrations at >180 min). Note that the relatively short-term stimulation of MPS (≈ 1.5 h), despite the continued AA availability, suggests that continuous intravenous nutrition is likely to result in the overprovision of AAs for protein synthesis.

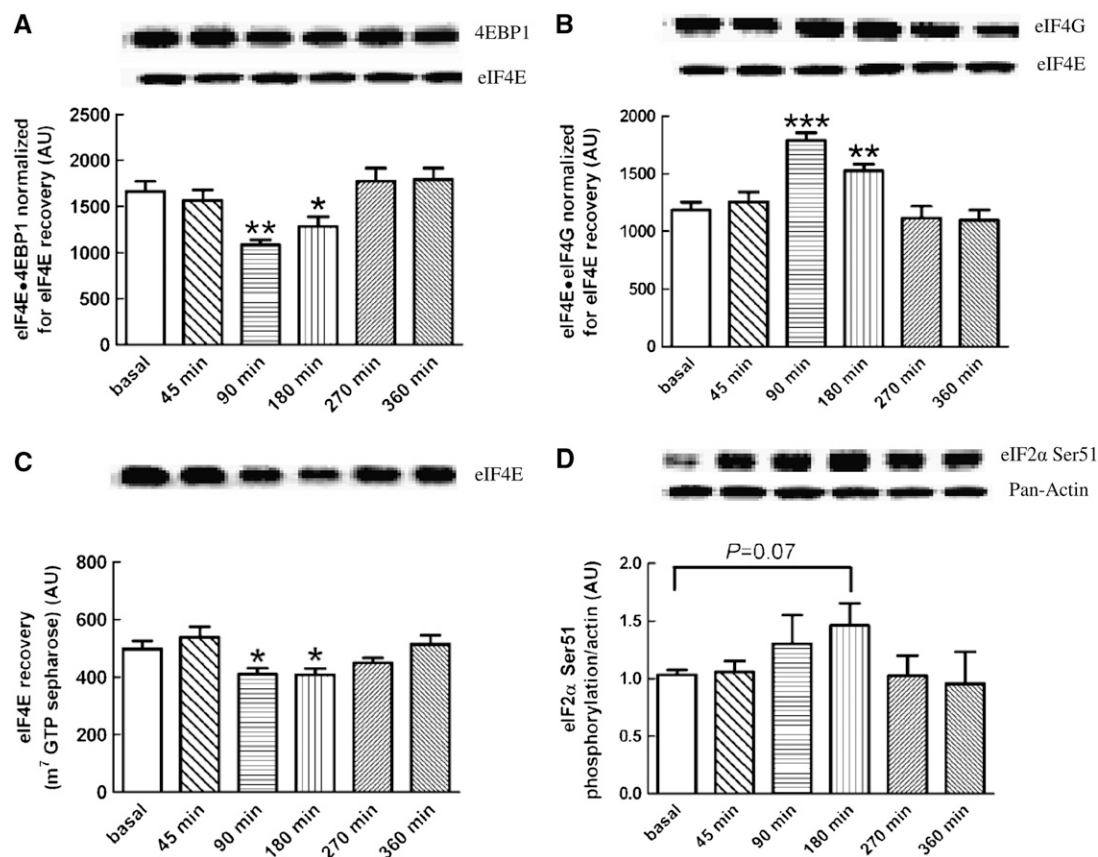


FIGURE 7. Eukaryotic initiation factor (eIF)-4F and eIF2 α analyses. Mean (\pm SEM) protein interactions assessed from eIF4E immunoprecipitates of eIF4E-4E-binding protein 1 (4EBP1) (A), eIF4E-eIF4G (B), total recovery of eIF4E from 7-methyl conjugated GTP sepharose (C), and phosphorylation of eIF2 α (D); $n = 8$. ****Significant increase from postabsorptive values in signaling protein phosphorylation (repeated-measures ANOVA with Tukey's post hoc test): * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. AU, arbitrary units; Ser, serine.

The notion that mTORC1 signaling regulates increases in MPS in response to anabolic stimuli such as exercise (25, 26) and EAAs (7, 11, 12) is widely accepted. In agreement with our results, Fujita et al (11) reported increases in S6K1 and 4EBP1 phosphorylation ≈ 1 h after a meal containing ≈ 20 g EAAs to young men. Nevertheless, because we did not observe an increased mTOR phosphorylation at Ser2448, despite increases in S6K1 and 4EBP1 phosphorylation after subjects were fed EAAs, suggests that such putative proximal regulators (eg, recombination-activating G proteins and mitogen-activated protein kinase kinase kinase 3) control mTORC1 via means independent of this site, perhaps via the regulating raptor-mTOR interaction (27) or phosphorylation of another site, such as Thr2446 (28). Also, despite being a Ser2448 kinase, S6K1 phosphorylation at Thr389 is reportedly insufficient for Ser2448 phosphorylation in vivo (29), at least in response to a single oral protein bolus. Furthermore, the mutation of Ser2448 to alanine does not affect mTORC1 kinase activity (30), and thus, Ser 2448 phosphorylation may not be a crucial event in regulating mTORC1 activity.

To discriminate regulatory from nonregulatory phosphorylation events, it is essential to make measurements over a time course that will match changes in MPS (encompassing both the upward and downward curves) with those of the putative regulatory signals. For example, it would be expected that an increase in those signals that preceded a measured incorporation of labeled leucine into muscle protein could be important in the response of

MPS to AA availability, especially as changes in regulatory signals (ie, phosphorylation) would precede the biological response (ie, increased MPS). For the first time to our knowledge, we provide these data in response to oral protein bolus in human beings, and at least for the increase in MPS, our data support a role for PKB and mTORC1 signaling. For example, the activity of PKB and eIF4G preceded that of MPS and increased initially by 45 min. At least for PKB, it is likely that the increase was insulin mediated because it mirrored the rise and fall in plasma insulin concentrations, and EAA effects are downstream of proximal insulin-signaling elements (ie, AAs do not activate phosphoinositide 3-kinase or PKB (31)). The fact that eIF4G phosphorylation preceded other indexes of mTORC1 signaling may indicate that its activity is PKB dependent or independent of mTORC1 signaling. Support for this latter proposition may be found in work that showed that dose responses in MPS responses to leucine were closely reflected by the amplitude of eIF4G, but not by S6K1 and 4EBP1 phosphorylation (32). In addition to the sustained PKB activity and eIF4G phosphorylation for up to 90 min, phosphorylation of 2 of the best-characterized mTORC1 substrates, S6K1 and 4EBP1, were increased by 90 min, which suggests that their activity may be required for sustaining increased MPS rather than regulating the initial increase alone.

What of the decline in MPS? We found much less of an explanation for this because, aside from PKB, the phosphorylation of mTORC1 substrates (ie, S6K1, 4EBP1 phosphorylation, and

eIF4E-eIF4G) remained elevated long after the rates of MPS had returned to baseline, which probably explains previous reports of phosphorylation increases persisting beyond 2 h (7, 12), despite the previously unexpected decline by these times of the rate of MPS. Moreover this phenomenon of anabolic signaling outlasting the MPS response has also been recently reported in neonatal pigs for eIF4F (33). One possible explanation could be that mRNA translation was still active, but the resulting newly synthesized polypeptides were degraded before we detected their incorporation into muscle protein, possibly by way of endoplasmic reticulum-associated degradation (34). Indeed, we may have some provisional evidence of this. For instance, eIF4E has only a single binding site for 7-methyl conjugated GTP (35); thus, only eIF4E not associated with mRNA is isolated by using this technique. Therefore, because the recovery of eIF4E by using 7-methyl conjugated GTP sepharose was significantly reduced when using this technique, this may signify that eIF4E was preferentially engaged in the binding of mRNA to the 40S ribosome (36). If this were the case, it is tempting to speculate that the raised concentrations of either or both plasma and intramuscular AAs were responsible for the continued stimulation and that another signal may exist to essentially override AA-induced signaling. But what could this be, and how would it signal? Increases in endoplasmic reticulum stress have been noted in response to an excess nutrient supply (37, 38), and we observed an induction of phosphorylation on the endoplasmic reticulum-stress responsive kinase eIF2 α 180 min after oral protein bolus when AA concentrations were still high. This may explain the reduction in MPS back to baseline rates through its inhibition of eIF2B activity (which reduces the exchange of eIF2-GDP to eIF2-GTP that is required to bind and deliver initiator methyl-transfer RNA_i to the translational machinery) and could also lead to an increase in endoplasmic reticulum-associated degradation. These possibilities require further substantiation.

In conclusion, mTORC1 signaling may control the increase in MPS after protein ingestion with the caveat that, if these signals indeed regulate increases in MPS to EAA, the functional significance of their outlasting MPS responses remains unexplained. Moreover, the short-term stimulation of MPS by AAs (at \approx 1.5 h) suggests that optimal clinical strategies should involve pulse rather than continuous supply of AA.

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