Time-dependent regulation of postprandial muscle protein synthesis rates after milk protein ingestion in young men

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Running head: Time course of postprandial muscle protein synthesis

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Abbreviations used:

AA,	amino acid
AKT,	protein kinase B;
FSR,	fractional synthetic rate
GC-MS,	gas chromatography-mass spectrometry
GC-P-IRMS	gas chromatography-pyrolysis-isotope mass spectrometry
LAT1,	large neutral amino acids transporter small subunit 1
LC-MS-MS,	liquid chromatography-tandem mass spectrometry;
R_a,	rate of appearance;
R_d,	rate of appearance;
MPE,	mole percent excess;
MPS,	muscle protein synthesis;
mTORC1,	mammalian target of rapamycin complex 1;
p70S6K,	70 kDa S6 protein kinase.

ABSTRACT

The anabolic action of 'fast' whey protein on the regulation of postprandial muscle protein synthesis has been established to be short-lived in healthy young adults. We assessed the time course of anabolic signaling activation and stimulation of myofibrillar protein synthesis rates (MPS) after ingestion of a food source that represents a more typical meal-induced pattern of aminoacidemia. Seven young men (age: 22±1 y) underwent repeated blood and biopsy sampling during primed continuous L-[ring-$^{2}H_5$]phenylalanine and L-[1-^{13}C]leucine tracer infusions, and ingested 38 g of L-[1-^{13}C]phenylalanine- and L-[1-^{13}C]leucine-labeled milk protein concentrate. A total of ~27±4% (~10 g) and ~31±1% (~12 g) of dietary protein-derived amino acids were released in circulation between 0-120 min and 120-300 min of the postprandial period, respectively. L-[ring-$^{2}H_5$]phenylalanine-based MPS increased above basal (0.025±0.008%·h^{-1}) by ~75% (0.043±0.009 %·h^{-1}; *P*=0.05) between 0-120 min and by ~86% (0.046±0.004 %·h^{-1}; *P*=0.02) between 120-300 min, respectively. L-[1-^{13}C]leucine-based MPS increased above basal (0.027±0.002%·h^{-1}) by ~72% (0.051±0.016 %·h^{-1}; *P*=0.10) between 0-120 min and by ~62% (0.047±0.004 %·h^{-1}; *P*=0.001) between 120-300 min, respectively. Myofibrillar protein-bound L-[1-^{13}C]phenylalanine increased over time (*P*<0.001) and equaled 0.004±0.001, 0.008±0.002, 0.017±0.004, and 0.020±0.003 mole percent excess at 60, 120, 180, and 300 min of the postprandial period, respectively. Milk protein ingestion increased mTORC1 phosphorylation at 120, 180, and 300 min of the postprandial period (all *P*<0.05). Our results show that ingestion of 38 g of milk protein results in sustained increases in MPS throughout a 5 h postprandial period in healthy young men.

23 **NEW & NOTEWORTHY**

24 The stimulation of muscle protein synthesis after whey protein ingestion is short-lived due to its
25 transient systemic appearance of amino acids. Our study characterized the muscle anabolic
26 response to a protein source that results in a more gradual release of amino acids into circulation.
27 Our work demonstrates that a sustained increase in postprandial plasma amino acid availability
28 after milk protein ingestion results in a prolonged stimulation of muscle protein synthesis rates in
29 healthy young men.

INTRODUCTION

Several studies have shown that protein ingestion elevates circulating amino acid availability to stimulate muscle protein synthesis rates in healthy adults (9, 11, 18, 23, 29, 30). This work defined dietary protein as a main anabolic stimulus to human skeletal muscle tissue. Less attention has been given to the time course of stimulation of muscle protein synthesis rates in response to elevated plasma amino acid availability. It has been previously shown that the ingestion of whey protein isolate stimulates a transient increase (ranging from ~45-120 min) in postprandial muscle protein synthesis rates before rapidly returning to baseline values, despite a prolonged elevation in plasma amino acid availability during the ensuing postprandial period (2, 21). This short-lived stimulation of the postprandial muscle protein synthetic response after whey protein ingestion has since been referred to as the "muscle-full" effect (2).

What is noteworthy, however, is that the postprandial plasma amino acid profile after whey protein ingestion (5, 23, 25) is unique when compared to other isolated protein sources such as casein or soy (26) as well as whole food sources of protein such as eggs or beef (9, 28). Specifically, the ingestion of whey, due to its solubility, results in high and transient appearance pattern of amino acids into circulation (2, 21, 23), which likely instigates the muscle-full phenomenon. Hence, it is relevant to define the time-dependent regulation of postprandial muscle protein synthesis rates after the ingestion of other types of protein sources with a more gradual and sustained release of dietary protein-derived amino acids into circulation when compared to 'fast' digesting whey protein. Such information can be utilized when developing anabolic feeding strategies in the practice of clinical or performance nutrition.

Therefore, the purpose of this work was to assess the relationship between dietary protein-derived amino acid availability and the subsequent time-dependent regulation of muscle protein synthesis rates after the ingestion of milk protein concentrate — containing both 'fast' whey and 'slow casein' as part of its protein matrix. To do this, we applied continuous L-[*ring*-$^{2}H_{5}$]phenylalanine and L-[1-^{13}C]leucine tracer infusion combined with the oral administration of intrinsically L-[1-^{13}C]phenylalanine and L-[1-^{13}C]leucine-labeled milk protein and repeated muscle biopsy sampling in healthy young men. This approach allowed for determination of the temporal pattern of dietary protein-derived amino acid release in the circulation, the stimulation of postprandial muscle protein synthesis rates as well as the utilization of the dietary protein-derived amino acids for *de novo* muscle protein synthesis throughout a 5-h postprandial period (8). Milk protein concentrate contains a combination of "fast" whey and "slow" casein protein fractions, and small amounts of carbohydrate and fats as part of its food matrix. As such, the ingestion of milk protein provides a more gradual release of dietary protein-derived amino acids throughout the postprandial phase when compared to the ingestion of free amino acid or whey protein (5, 23). We hypothesized that the ingestion of 38 g of milk protein would result in a sustained activation of anabolic signaling, a sustained stimulation of postprandial muscle protein synthesis rates, and a progressive accumulation of dietary protein derived amino acids for *de novo* muscle protein accretion throughout the 0-300 min postprandial period in healthy, young men.

METHODS

Participants and ethical approval

Seven healthy, young men (age: 22±1 y) volunteered to participate in this study. All participants were deemed healthy based on their response to a routine medical screening questionnaire. Volunteers had no history of participating in past stable isotope amino acid tracer experiments. Participants' characteristics are presented in **Table 1**. All participants were informed about the experimental procedures to be used, the purpose of the study, and all potential risks before giving written consent. The study conformed to all standards for the use of human participants in research as outlined in the Helsinki Declaration and approved by the local Institutional Review Board at the University of Illinois at Urbana-Champaign (IRB # 14234).

Experimental protocol

Participants reported to the laboratory at 0700 h after an overnight fast and having refrained from strenuous physical activity for at least 3 d prior to the experimental trial. A Teflon catheter was inserted into a heated dorsal hand vein for repeated arterialized blood sampling and remained patent by a 0.9% saline drip. After taking a baseline blood sample (t= −180 min), the plasma phenylalanine, tyrosine, and leucine pools were primed with a single dose of L-[*ring*-$^{2}H_5$]phenylalanine (2.0 μmol·kg), L-[*ring*-3,5-$^{2}H_2$]tyrosine (0.615 μmol·kg), and L-[1-^{13}C]leucine (4.0 μmol·kg), after which a continuous L-[*ring*-$^{2}H_5$]phenylalanine (0.05 μmol·kg^{-1}·min), L-[*ring*-3,5-$^{2}H_2$]tyrosine (0.015 μmol·kg·min), and L-[1-^{13}C]leucine (0.10 μmol·kg·min) intravenous infusion was initiated (t= −180 min) and maintained over the experimental infusion

trial. Muscle biopsy samples were collected before (t= −120 and 0 min) and after (t= 60, 120, 180 and 300 min) the ingestion of 38 g of intrinsically L-[1-^{13}C]phenylalanine and L-[1-^{13}C]leucine-labeled milk protein concentrate dissolved in 300 mL of water. Biopsies were collected from the middle region of the *vastus lateralis* (approximately 15 cm above the patella) with a Bergström needle that was modified for manual suction under local anesthesia (18). Muscle samples were freed from any blood, fat, and visible connective tissue and immediately frozen in liquid nitrogen prior to storage at −80°C until further analysis. Blood samples were collected in EDTA containing tubes before (t= −180, −120, and −60 min) and after milk protein ingestion (t= 30, 60, 90, 120, 180, 240 and 300 min). The blood samples were immediately analyzed for whole blood glucose concentrations (2300 Stat Plus, YSI Life Sciences, Springs, OH) and centrifuged at 3000*g* for 10 min at 4°C. The plasma samples were subsequently stored at -20°C for future analysis.

Intrinsically labeled milk protein

Intrinsically L-[1-^{13}C]phenylalanine and L-[1-^{13}C]leucine labeled milk protein concentrate was obtained by infusing L-[1-^{13}C]phenylalanine and L-[1-^{13}C]leucine into a lactating Holstein cow, collecting the milk, and purifying the milk protein concentrate as previously described (10, 24, 27). The L-[1-^{13}C]phenylalanine and L-[1-^{13}C]leucine enrichments in the milk protein concentrate were measured by GC-MS (Agilent 6890N GC coupled with a 5973 inert MDS; Little Falls, DE) and averaged 38.3 and 10.8 mole percent excess (MPE), respectively. The macronutrient composition and energy of the milk protein beverage provided to participants was

38 g protein (3.46 g leucine), 4.17 g carbohydrate, and 1.4 g fat. The milk protein met all chemical and bacteriologic specifications for human consumption.

Plasma analyses

Plasma insulin concentrations were determined using a commercially available enzyme-linked immunosorbent assays (Alpco Diagnostics; Salem, NH). Plasma amino acid concentrations and enrichments were determined by GC-MS (Agilent 7890A GC/5975C; MSD, Little Falls, DE) as previously described (16).

Muscle analyses

Myofibrillar proteins were extracted from ~50 mg of wet muscle by hand-homogenizing in ice-cold homogenization buffer (10 μL·mg) containing phosphatase- (Roche PhosSTOP™) and protease inhibitors (Roche cOmplete™ Protease Inhibitor) using a Teflon pestle as previously described (29). For measurement of muscle protein-bound L-[*ring*-$^{2}H_{5}$]-phenylalanine, L-[1-^{13}C]phenylalanine, and L-[1-^{13}C]leucine enrichment, the eluate was dried and the purified amino acids were derivatized to their N(O,S)-ethoxycarbonyl ethyl esters. The derivatized L-[*ring*-$^{2}H_{5}$]phenylalanine samples were measured using a gas chromatography-isotope ratio mass spectrometer (MAT 253; Thermo Fisher Scientific, Bremen, Germany) equipped with a pyrolysis oven (GC-P-IRMS) and a 60 m DB-17MS column and 5 m precolumn (No. 122–4762; Agilent) and GC-Isolink. Ion masses 1 and 2 were monitored to determine the $^{2}H/^{1}H$ ratios of muscle protein bound phenylalanine. The derivatized L-[1-^{13}C]phenylalanine and L-[1-^{13}C]leucine samples were measured using a gas chromatography-isotope ratio mass spectrometer (Finnigan MAT 252; Thermo Fisher Scientific, Bremen, Germany) equipped with a Ultra I GC-

column (no. 19091A-112; Hewlett-Packard, Palo Alto, CA) and combustion interface II (GC-C-IRMS). Ion masses 44, 45, and 46 were monitored for $^{13}C/^{12}C$ phenylalanine and leucine, respectively. By establishing the relationship between the enrichment of a series of L-[1-^{13}C]phenylalanine, L-[1-^{13}C]leucine, and L-[*ring*-$^{2}H_5$]phenylalanine standards of variable enrichment and the enrichment of the *N(O,S)*-ethoxycarbonyl ethyl esters of these standards, the muscle-protein-bound enrichment of phenylalanine and leucine was determined.

Muscle intracellular free amino acids were extracted from a separate piece of wet muscle (~30 mg) using a Teflon-coated pestle as described previously (29). The muscle intracellular leucine and phenylalanine ^{13}C and ^{2}H enrichments were determined by multiple reaction monitoring (MRM) at *m/z* 132.0 → 86.0 and 133.0 → 87.0 for unlabeled and labeled L-[1-^{13}C]leucine and *m/z* 166.0 → 103.0, 167.0 → 104.0 and 171.0 → 106.0 for unlabeled and labeled (L-[1-^{13}C] and *ring*-$^{2}H_5$)phenylalanine, respectively. Software Analyst 1.6.2 was used for data acquisition and analysis.

Western blotting

A portion of whole muscle homogenates isolated during the myofibrillar protein extractions was used for Western blotting analysis, and was described and validated previously (3). Protein content of the homogenates was determined by Bradford Assay (Bio-Rad Laboratories, Hercules, CA) and then equal amounts of protein were separated by SDS-PAGE before being transferred to polyvinyl difluoride membranes for blotting. After blocking, membranes were incubated in primary antibodies overnight at 4^{o}C to determine the phosphorylation status and total protein content of protein kinase B (Akt) at Ser^{473}, mammalian target of rapamycin complex 1

(mTORC1) at Ser^{2448}, 70 kDa S6 protein kinase 1 (p70S6K1) at Thr^{389} (Cell Signaling Technology, Danvers, MA) and large neutral amino acid transporter (LAT1 SLC7A5) (total protein content only) (Bioss Antibodies, Woburn, MA). Membranes from the respective proteins were then incubated with appropriate secondary antibodies and protein content was detected using West Femto Maximum Sensitivity substrate (SuperSignal, Thermo Scientific, Waltham, MA) and the ChemiDoc-It^2 Imaging System (UVP, Upland, CA). After detection of phosphorylated proteins, membranes were stripped with western blot stripping buffer (Restore, Thermo Scientific, USA) and re-incubated with antibodies against total protein (Cell Signaling Technology, Danvers, MA). Western blot data were normalized to an internal control (α-tubulin). Bands were quantified using ImageJ software (NIH), normalized to a control sample run on each blot to account for inter-blot variability, and expressed as fold change from basal.

Calculations

Ingestion of L-[1-^{13}C]phenylalanine-labeled protein, intravenous infusion of L-[*ring*-$^2H_5$]phenylalanine and L-[*ring*-3,5-$^2H_2$]tyrosine, and arterialized blood sampling were used to assess whole-body amino acid kinetics in non-steady state conditions. Total, exogenous, and endogenous rate of appearance (R_a), plasma availability of dietary protein-derived phenylalanine (i.e., the fraction of dietary protein-derived phenylalanine that appeared in the systemic circulation, Phe_{plasma}), were calculated using modified Steele's equations (6, 13) as described previously (19). Furthermore, total rate of phenylalanine disappearance (R_d), utilization of phenylalanine for protein synthesis, and phenylalanine hydroxylation (first step of phenylalanine conversion to tyrosine) were calculated (19). Myofibrillar protein fractional synthetic rates (FSR)

179 were calculated using standard precursor - product methods by dividing the increment in tracer
180 enrichment in the myofibrillar protein fraction by the enrichment of the plasma or intracellular
181 precursor pools over time as described previously (8). For basal muscle protein FSR, muscle
182 biopsies at t= −120 and 0 min were used, and for postprandial FSR, biopsies at t= 60, 120, 180
183 and 300 min were used to calculate FSR.
184
185 **Statistics**
186 Differences in plasma and muscle time curves were tested using one-way repeated-measures
187 ANOVA (time). For all analysis, when statistically significant time effects were observed
188 Fisher's Least Significant Difference (LSD) tests were performed to locate differences.
189 Differences were considered statistically significant at P<0.05. All calculations were performed
190 using IBM SPSS Statistics (version 25, Chicago, IL). All data are expressed as mean ± SEMs.
191
192 **RESULTS**
193
194 **Plasma parameters**
195 Plasma parameters are shown in **Table 2**. Plasma phenylalanine, tyrosine, and leucine
196 concentrations increased rapidly after milk protein ingestion (all time effect: P<0.01) and
197 remained elevated above basal values (t= 0 min) during the entire post-prandial phase (all time
198 points, P<0.05). Peak concentrations for all three amino acids were observed at 30 min after
199 milk protein ingestion with values of 97 ± 5, 116 ± 10, and 335 ± 32 $\mu mol \cdot L^{-1}$ for phenylalanine,

tyrosine, and leucine, respectively. Plasma glucose and insulin concentrations increased transiently at 30 min after milk protein ingestion (both time effect: $P<0.01$).

Plasma amino acid enrichments

Plasma L-[1-^{13}C]phenylalanine enrichments (**Figure 1A**) increased rapidly after milk protein ingestion (time effect: $P<0.01$) and remained elevated above basal values (t= 0 min) during the 300 min post-prandial phase (all time points, $P<0.05$). Plasma L-[*ring*-$^{2}H_5$]-phenylalanine (infused tracer) enrichments (**Figure 1B**) decreased after milk protein ingestion (time effect: $P<0.01$) and were reduced below basal values until 60 min of the postprandial period (all time points, $P<0.05$). Plasma L-[*ring*-3,5-$^{2}H_2$]tyrosine (**Figure 1C**) decreased after protein ingestion (time effect: $P<0.01$) and remained suppressed below basal values (t= 0 min) until 120 min after protein ingestion (all time points, $P<0.05$). Plasma L-[1-^{13}C]leucine (**Figure 1D**) enrichments increased following protein ingestion (time effect: $P<0.01$) at 30 min and remained steady during the remaining post-prandial phase.

Muscle free amino acid enrichments

Muscle tissue free L-[1-^{13}C]-phenylalanine enrichments increased after milk protein ingestion (time effect: $P<0.01$), reaching peak values at t= 120 min (6.1 ± 0.6 MPE), and remained elevated above basal values (t= 0 min) during the entire post-prandial phase (all time points, $P<0.05$) (**Figure 1A**). Muscle tissue free L-[*ring*-$^{2}H_5$]-phenylalanine enrichments decreased after milk protein ingestion (time effect: $P<0.03$) and were suppressed below basal values (t= 0 min) at 60 min of the postprandial period ($P=0.02$) (**Figure 1B**). Muscle tissue free L-[1-

222 ^{13}C]leucine enrichments increased after protein ingestion (time effect: $P<0.01$) at 60 min after
223 milk protein ingestion ($P=0.04$) remained steady during the post-prandial phase from 120 min
224 onwards (**Figure 1C**).

Plasma amino acid kinetics

Exogenous phenylalanine rates of appearance (representing the appearance of dietary protein-derived phenylalanine into the circulation) (**Figure 2A**) increased after milk protein ingestion (time effect: $P<0.01$) and remained elevated above basal values (t=0 min) during the entire post-prandial phase (all time points, $P<0.01$). Peak plasma exogenous phenylalanine rates of appearance were observed at 30 min after milk protein ingestion and reached a value of 313 ± 48 nmol phenylalanine·kg^{-1}·min^{-1}. The cumulative amount of dietary protein-derived phenylalanine that appeared in circulation during 0-60, 0-120, 0-180, and 0-240 and 0-300 min was 13±2, 27±4, 38±4, 48±4 and 58±4 %, respectively (**Figure 2B)**.

Total plasma phenylalanine appearance and disappearance rates increased after milk protein ingestion (all time effect: $P<0.01$) and remained elevated above basal values (t= 0 min) at 180 min of the postprandial period (all time points, $P<0.05$). Whole body protein breakdown (represented as endogenous phenylalanine rates of appearance) decreased after milk protein ingestion (time effect: $P<0.01$) and remained suppressed below basal values until 300 min of the postprandial phase. Whole body protein oxidation rates (represented as phenylalanine hydroxylation) increased after milk protein ingestion (time effect: $P<0.01$) and remained elevated above basal values (t=0 min) during the entire post-prandial phase (all time points, $P<0.05$). Whole body protein synthesis rates (represented as total phenylalanine rates of disappearance – phenylalanine hydroxylation) increased after protein ingestion (time effect: $P<0.01$). Consequently, whole body net protein balance (represented as synthesis – breakdown) increased after protein ingestion (time effect: $P<0.01$) and remained elevated above basal values (t= 0 min) during the entire post-prandial phase (all time points, $P<0.01$).

247 **Muscle anabolic signaling**

248 Milk protein ingestion did not modulate relative muscle LAT1 protein content (**Figure 3A**)
249 during the postprandial period (time effect: P=0.53). AKT phosphorylation (**Figure 3B**) tended
250 to increase after milk protein ingestion (time effect: P=0.09). mTORC1 phosphorylation (**Figure**
251 **3C**) increased after milk protein ingestion (time effect: P=0.02) and was significantly elevated
252 above basal values (t= 0 min) between 120-300 min of the postprandial period (all time points,
253 P<0.01). There was no difference in p70S6K phosphorylation (**Figure 3D**) after milk protein
254 ingestion from basal (time effect: P=0.11)

255

256 **Myofibrillar protein synthesis**

257 The temporal change in myofibrillar bound protein enrichments are shown in **Table 3**. With the
258 use of plasma L-[ring-$^2H_5$]phenylalanine enrichments as the precursor, postprandial myofibrillar
259 protein synthesis rates increased above basal values (0.025±0.008 %·h^{-1}) (time effect: P=0.013)
260 between 0-120 (0.043±0.009 %·h^{-1}), 120-300 (0.046±0.004 %·h^{-1}), and 0-300 min
261 (0.045±0.004 %·h^{-1}) (all timepoints, P<0.05) (**Figure 4A**) (0-300 min data not shown in figure).
262 No differences were observed between 0-120 and 120-300 min postprandial FSRs (P=0.76).
263 Postprandial plasma L-[ring-$^2H_5$]phenylalanine based muscle protein FSRs expressed at 0-60,
264 120-180, 120-180 and 180-300 min intervals were 0.043±0.010 %·h^{-1}, 0.043±0.010 %·h^{-1},
265 0.045±0.015 %·h^{-1}, and 0.045±0.007 %·h^{-1}, respectively (**Figure 4B**) (time effect: P=0.46). FSRs
266 calculated with muscle free L-[ring-$^2H_5$]phenylalanine enrichments were 0.105±0.031 %·h^{-1};
267 0.154±0.037 %·h^{-1}; 0.150±0.032 %·h^{-1}; 0.162±0.050 %·h^{-1}; and 0.167±0.027 %·h^{-1} at basal, and

268 between 0-60, 60-120, 120-180, and 180-300 min of the postprandial phase, respectively (time
269 effect: P=0.64).

270 With the use of plasma L-[1-^{13}C]leucine enrichments as the precursor, postprandial muscle
271 protein FSRs increased above basal values (0.027±0.002 %·h^{-1}) (time effect: P=0.018) between
272 120-300 (0.047±0.004 %·h^{-1}) and 0-300 min (0.049±0.007 %·h^{-1}) (all time points, P<0.05)
273 (**Figure 4C**). We observed a trend (P=0.10) for an increase in FSR above basal values between
274 0-120 min (0.051±0.016 %·h^{-1}). No differences were observed between 0-120- and 120-300-min
275 postprandial muscle protein FSRs (P=0.85). Postprandial plasma L-[1-^{13}C]leucine enrichment
276 based muscle protein FSRs expressed at 0-60, 120-180, 120-180 and 180-300 min were
277 0.065±0.015 %·h^{-1}, 0.035±0.015 %·h^{-1}, 0.067±0.018 %·h^{-1}, and 0.036±0.012 %·h^{-1}, respectively
278 (time effect: P=0.46) (**Figure 4D**). Muscle protein FSRs calculated with muscle free L-[1-
279 ^{13}C]leucine enrichments were 0.040±0.003 %·h^{-1}; 0.083±0.020 %·h^{-1}; 0.043±0.019 %·h^{-1};
280 0.077±0.022 %·h^{-1}; and 0.039±0.014 %·h^{-1} at basal, and between 0-60, 60-120, 120-180, and
281 180-300 min of the postprandial stage respectively (time effect: P=0.25).

282 Dietary protein-derived L-[1-^{13}C]-phenylalanine enrichment was detected in myofibrillar
283 proteins at 60 min (0.004±0.001 MPE). The muscle protein bound L-[1-^{13}C]-phenylalanine
284 enrichment progressively increased at 120 min (0.008±0.002 MPE) and 180 min (0.017±0.004
285 MPE) before a plateau was achieved at 300 min (0.020±0.003 MPE) of the postprandial phase
286 (**Figure 5**) (time effect: P<0.001). No differences were observed in the myofibrillar bound L-[1-
287 ^{13}C]-phenylalanine enrichments at the early (0-120 min) (0.008±0.002 MPE) or late postprandial
288 stage (120-300 min) (0.012±0.002 MPE; P=0.17).

289

290 **DISCUSSION**

291 Previous studies have used the administration of constant intravenous amino acid infusions or
292 whey protein ingestion to describe a latency and saturable postprandial muscle protein synthetic
293 response after elevated plasma amino acid availability (2, 4, 21). Here, we demonstrated that the
294 ingestion of 38 g of milk protein results in a rapid and sustained release of dietary protein
295 derived amino acids into circulation, thereby providing a prolonged exposure of exogenous
296 protein derived amino acids to the muscle during the entire 300 min postprandial phase (2, 21).
297 This pattern of aminoacidemia after milk protein ingestion resulted in a sustained stimulation of
298 muscle protein synthesis rates during the entire 5 h postprandial period. Likewise, dietary protein
299 derived amino acids are utilized by muscle throughout the early (0-120 min) and late (120-300
300 min) postprandial period as evidenced by the progressive increase in L-[1-^{13}C]-phenylalanine
301 incorporation into muscle protein.

302 A noteworthy aspect of our study was the use of intrinsically labeled protein method (24, 27),
303 which allowed us to quantify the postprandial release of dietary protein-derived amino acids into
304 the circulation and the subsequent time course of stimulation of muscle protein synthesis rates *in*
305 *vivo* in humans. We observed rapid protein digestion and amino acid absorption after ingesting
306 38 g milk protein, which resulted in ~13±2% (~4.8 g protein) of dietary protein-derived amino
307 acid becoming available into circulation within the first hour of the postprandial period (**Figure**
308 **2B**). Plasma amino acid availability peaked between 60-120 min, during which time ~14±1%
309 (~5.3 g) of ingested milk protein was released into circulation, and the milk protein-derived
310 amino acids continued to be released during the later postprandial period (**Figure 2**). This is
311 similar to the pattern of aminoacidemia observed after the ingestion of ample amounts of protein

312 contained in eggs, meat, or skim milk, which are other food sources commonly consumed within
313 a Western diet (3, 9, 29). In contrast, whey protein induces a rapid and transient pattern of
314 aminoacidemia (2, 21, 23) with the postprandial stimulation of muscle protein synthesis rates
315 only lasting 60-180 min (2, 21). Overall, the moderate and prolonged dietary protein derived
316 amino acid release into circulation and the sustained stimulation of muscle protein synthesis rates
317 after the ingestion of 38 g milk protein is likely more indicative of an anabolic response to whole
318 food ingestion (or mixed meal feeding) in comparison to whey protein ingestion or intravenous
319 amino acid infusions.

320 Importantly, the use of intrinsically L-[1-^{13}C]-phenylalanine labeled milk protein allowed us
321 to determine the utilization of dietary protein-derived amino acids for *de novo* muscle protein
322 accretion. This approach allowed for the first time the direct assessment of the meal-derived
323 amino acid accretion into muscle proteins in a time dependent manner (**Figure 5A**). We show
324 that dietary-derived amino acids were rapidly used for *de novo* myofibrillar protein synthesis as
325 evidenced by the increase in myofibrillar bound L-[1-^{13}C]-phenylalanine enrichment at 60 min of
326 the postprandial period. Moreover, dietary protein-derived amino acids are continuously used for
327 postprandial muscle protein accretion into the late phase of the postprandial period (**Figure 5B**).
328 Specifically, we show that ~2.9±0.6% (~1.1±0.3 g) of dietary protein-derived amino acids were
329 incorporated into *de novo* muscle protein within 0-120 min and ~4.2±0.9% (~1.6±0.3 g) of the
330 dietary amino acids were incorporated during the subsequent 120-300 min postprandial phase.

331 The mTORC1 pathway has been extensively studied as the nexus for nutrient-related
332 anabolic signals (i.e., elevated dietary amino acids as opposed to plasma insulin) that regulate the
333 postprandial stimulation of muscle protein synthesis rates in humans (2, 15, 21). Previously, we

have demonstrated that increases in postprandial mTORC1 phosphorylation events are often modest or undetectable after the ingestion of protein dense foods (i.e., eggs, pork, or skim milk) (3, 9, 29). We have generally attributed the diminished activation of the mTOR pathway in these prior studies to muscle biopsy timing issues that are often associated with study designs aimed at optimizing the measurement of muscle protein synthesis rates as opposed to capturing static snapshots of protein phosphorylation. We accounted for this issue in the present study by collecting muscle biospy samples more frequently throughout the postprandial period. With this approach, we show that the ingestion of milk protein increased mTORC1 phosphorylation on Ser2448 at 120 min, and that this response remained elevated throughout the subequent postprandial period (**Figure 3C**). This prolonged activation of mTOR supports the notion that the anabolic response to the ingestion of 38 g of milk protein is sustained throughout a 300 min postprandial period. It is worth acknowledging, however, that it has been suggested that mTORC1 phosporylation on Ser2448 may not be representative of mTORC1 activity, and that other targets are likely preferred (e.g., p70S6K) (14). In the present work, p70S6K and other molecular readouts linked to the mTORC1 pathway (p-Akt and total LAT1 protein content) did not change throughout the postprandial period (**Figure 3**). The absence of changes in the phosphorylated state of AKT and p70S6K in the current work are perhaps suggesting that modest anabolic signaling activation is sufficient to elicit changes in muscle protein synthesis rates when the postprandial release of dietary amino acids into circulation is more gradual. Past efforts have shown that there is a dose-dependent increase in the phosphorylation of AKT on Ser473 and p70S6K on Thr389 in response to increasing plasma insulin and amino acid concentrations (12, 17). However, the most robust changes in the phosporylation of anabolic signaling molecules,

such as p70S6K, are generally observed after the ingestion of free amino acids (15) or whey protein ingestion (2, 20), which demonstrate more rapid patterns of elevated aminoacidemia when compared to milk protein ingestion. Additionally, we may have been underpowered to detect subtle increases in the phosphorylated state of p70S6K, which was ~ 1.5 fold elevated above basal at 120 and 180 min of the postprandial phase, but did not reach statistical significance (time effect P=0.11).

A question raised by this study is: what is the significance of the sustained increase of muscle protein synthesis rates after the ingestion of protein-dense food? Firstly, we have established that the meal-induced stimulation of postprandial muscle protein synthesis rates is extended beyond the early period (>180 min), which is contrast to earlier observations (2, 21). Like past studies, our experiment was conducted in the morning with participants in the post-absorptive state. Whether this sustained postprandial muscle anabolism may only be pertinent to the first meal of the day (i.e., breakfast) with the anabolic sensitivity of muscle tissue to protein ingestion waning over the course of the day (i.e., breakfast>lunch>dinner) is currently unknown. Indeed, it was previously demonstrated that muscle protein synthesis rates can be maintained above post-absorptive values by the ingestion of whey protein every ~4 h during a 12 h experimental protocol (1). However, it is not possible to distinguish the anabolic potential of each individual meal from this study design. Future studies are clearly required to assess how the quantity and pattern of dietary protein intake within a mixed meal setting over the course of the day differentially modulates postprandial muscle protein synthesis rates, and ultimately influences daily net protein balance, to define if there is a most important protein meal of the day.

From a study design perspective, we applied repeated muscle biopsy sampling to assess the stimulation of muscle protein synthesis rates in an hourly fashion based on the infused tracers (**Figure 4B and D**). Our results, however, show that the postprandial muscle protein synthetic response was not statistically stimulated above basal values based on the hourly analysis regardless of the infused tracer (i.e., labeled leucine or phenylalanine). This finding contrasts with our assessment of muscle protein synthesis over the early (0-120 min) and late (120-300 min) postprandial phase. The disagreement between findings likely relates to the heterogeneity of the response between participants and analytical challenges in detecting changes in protein-bound enrichments when successive muscle biopsy samples are collected during short infusion time intervals. Hence, longer incorporation times (>1 h) are likely warranted when assessing the stimulation of postprandial muscle protein synthesis rates to protein dense food ingestion, especially when using smaller sample sizes.

In summary, we are the first to provide insight into the postprandial release of dietary amino acids into circulation and the subsequent regulation of postprandial muscle protein synthesis rates to a more slowly digested protein source than whey. We show that the ingestion of 38 g of milk protein results in a persistent 'anabolic drive' to skeletal muscle tissue as shown by the sustained increase in plasma amino acid availability, increased mTORC1 phosphorylation, and the stimulation of postprandial muscle protein synthesis rates during the early (0-120 min) and late (120-300 min) postprandial periods. Similarly, the utilization of dietary protein derived amino acids for *de novo* muscle protein accretion is rapid and persists into the late postprandial period in healthy young men. Future studies are required to determine if our results are relevant to all meals consumed in a day (e.g., lunch or dinner).

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403

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406

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413

414 **Author contributions**

415 The authors' responsibilities were as follows—SvV, LJCvL, and NAB: contributed to the
416 conception and the design of the experiment; all authors: contributed to collection, analysis, and
417 interpretation of data; SvV, AMH, LJCvL and NAB: contributed to drafting or revising
418 intellectual content of the manuscript and had primary responsibility for the final content; all
419 authors: read, edited, and approved the final version of the manuscript.

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FIGURE LEGENDS

FIGURE 1. Plasma (●) and muscle free (□) L-[1-^{13}C]phenylalanine- (A), L-[*ring*-$^{2}H_5$]phenylalanine- (B), L-[*ring*-3,5-$^{2}H_2$]tyrosine- (C), and L-[1-^{13}C]leucine (D) enrichments (MPE) in the basal state and after ingestion of milk protein (38 g) in healthy young men (*n*=7). Dashed line refers to protein ingestion. Data were analyzed with one-way repeated-measures ANOVA (time). The LSD test was used to locate differences between means when significance was observed. *Different from basal (t=0 min); $^{\#}$Different from 0-60 min (P<0.05). Data are mean ± SEM. MPE, mole percent excess.

FIGURE 2. Exogenous phenylalanine R_a (A) and cumulative dietary-derived Phe_{plasma} (%) (B) in the basal state (not shown for Phe_{plasma}) and after ingestion of milk protein (38 g) in healthy young men (*n*=7). Dashed line refers to protein ingestion. Data were analyzed with one-way repeated-measures ANOVA (time). The LSD test was used to locate differences between means when significance was observed. Exogenous R_a (A), cumulative dietary-derived Phe_{plasma} (B): all time effect, P<0.01. *Different from basal (t=0 min) (P<0.05). Means without a common letter significantly differ. Data are mean ± SEM. R_a, rate of appearance.

FIGURE 3. Phosphorylation status of LAT1 (A), AKT^{Ser473} (B), $mTOR^{Ser2448}$ (C), $P70S6K1^{Thr389}$ (D) in the basal state (t=0 min; grey bars) and after ingestion of milk protein (38 g; white bars) in healthy young men (*n*=7). Data were analyzed with one-way repeated-measures ANOVA (time). The LSD test was used to locate differences between means when significance was observed. *Different from basal (t=0 min) (P<0.05). Data are mean ± SEM. AKT, protein kinase B; LAT1,

Large neutral amino acid transporter small subunit 1; mTORC1, mammalian target of rapamycin complex 1; 70 kDa S6 protein kinase 1.

FIGURE 4. Myofibrillar protein L-[*ring*-$^{2}H_5$]phenylalanine (A and B) and L-[1-^{13}C]leucine FSRs (C and D)as %·h^{-1} in the basal state (grey bars) and after ingestion of milk protein (38 g; white bars) in healthy young men (*n*=7) using the plasma enrichments as the precursor pool. Data were analyzed with one-way repeated-measures ANOVA (time). The LSD test was used to locate differences between means when significance was observed. *Different from basal (t= −120-0 min) (*P*<0.05). Data are mean ± SEM. FSR, fractional synthesis rates.

FIGURE 5. Myofibrillar protein-bound L-[1-^{13}C]phenylalanine enrichment (MPE) after ingestion of milk protein (38 g) in healthy young men (n=7). Data were analyzed with one-way repeated-measures ANOVA (time). The LSD test was used to locate differences between means when significance was observed. Means without a common letter significantly differ (*P*<0.05). Data are mean ± SEM MPE, mole percent excess.

FIGURE 1

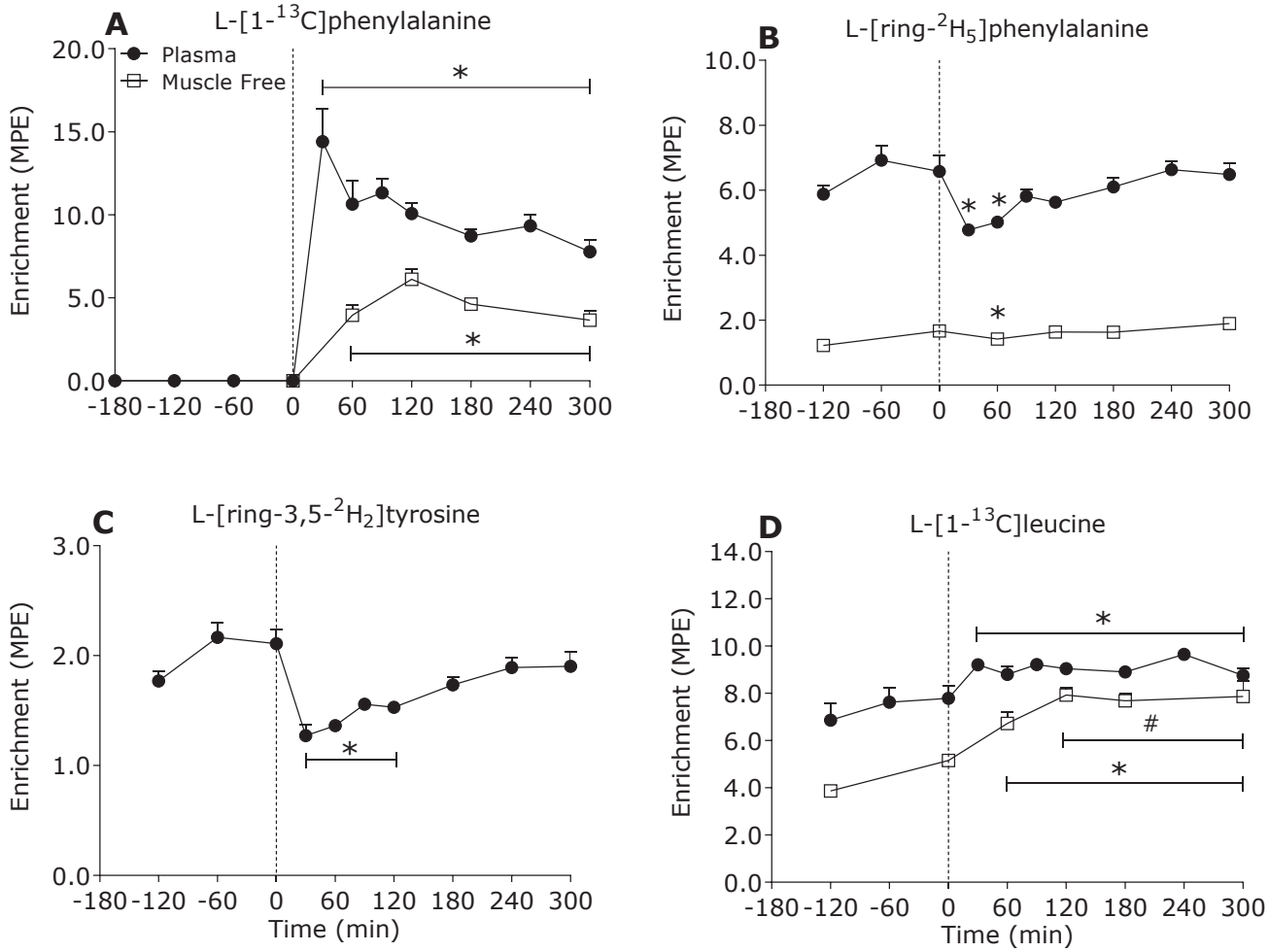

FIGURE 2

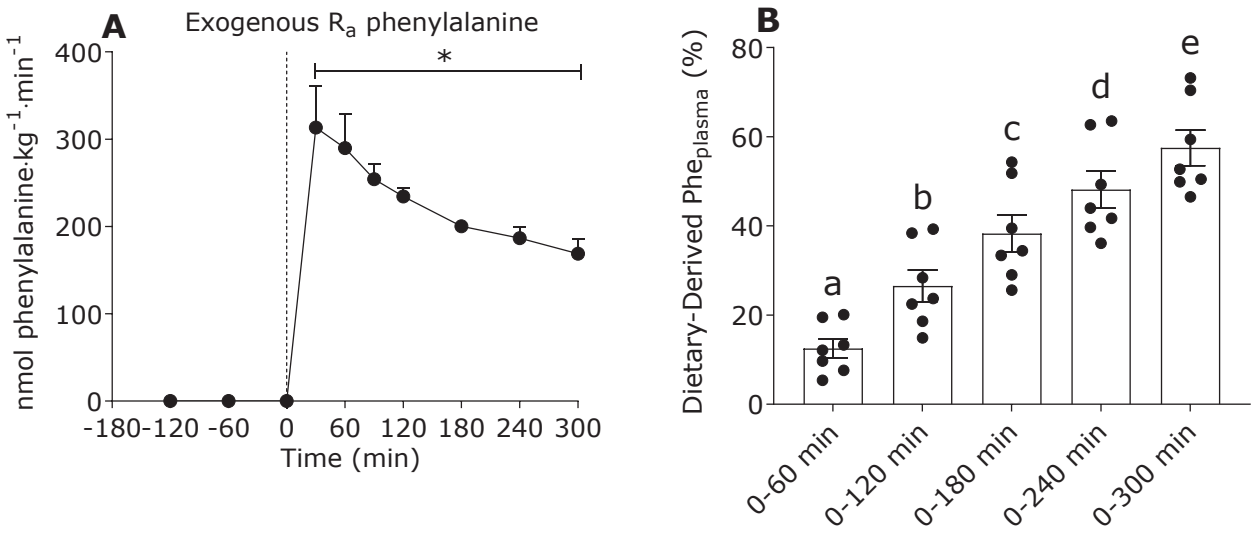

FIGURE 3

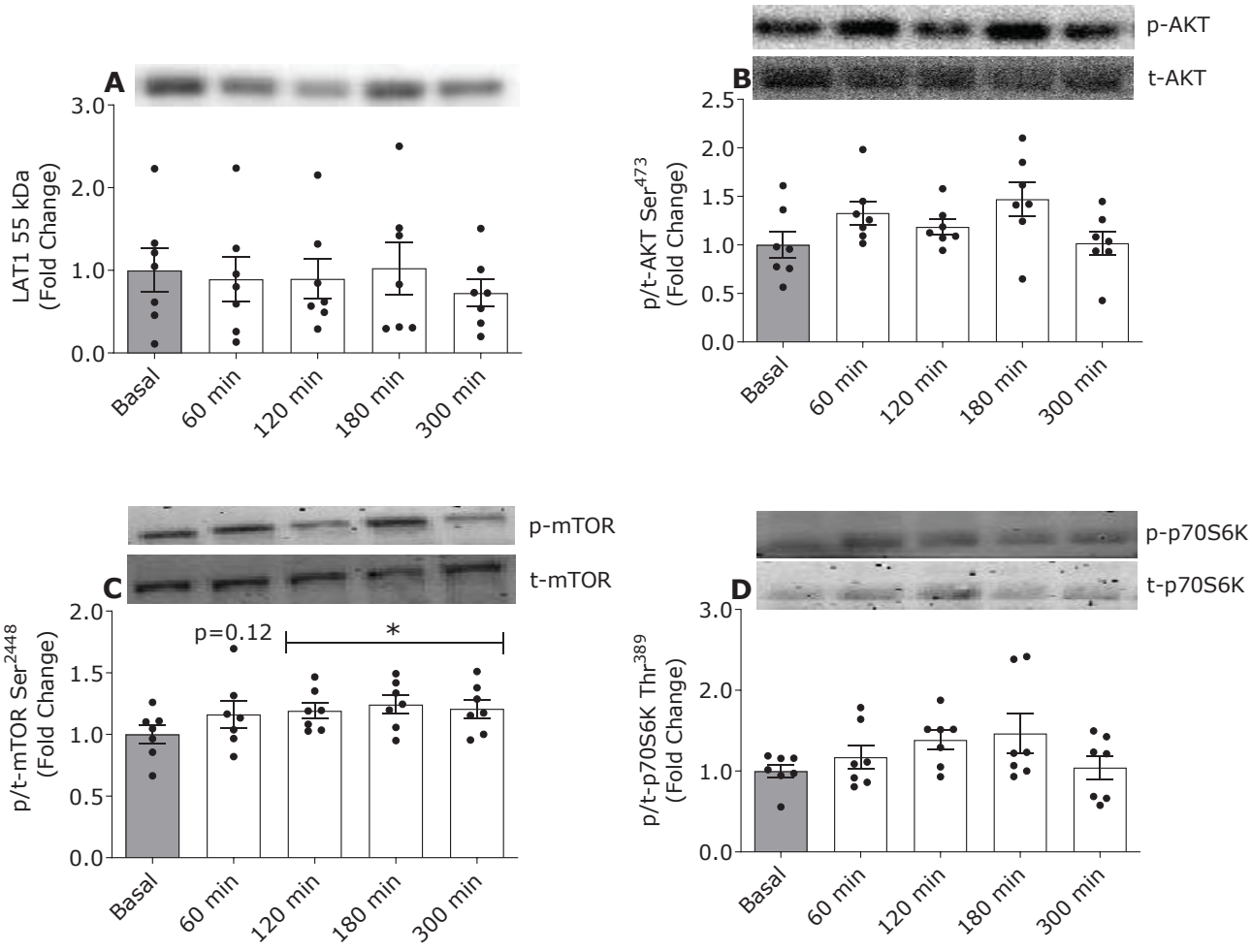

FIGURE 4

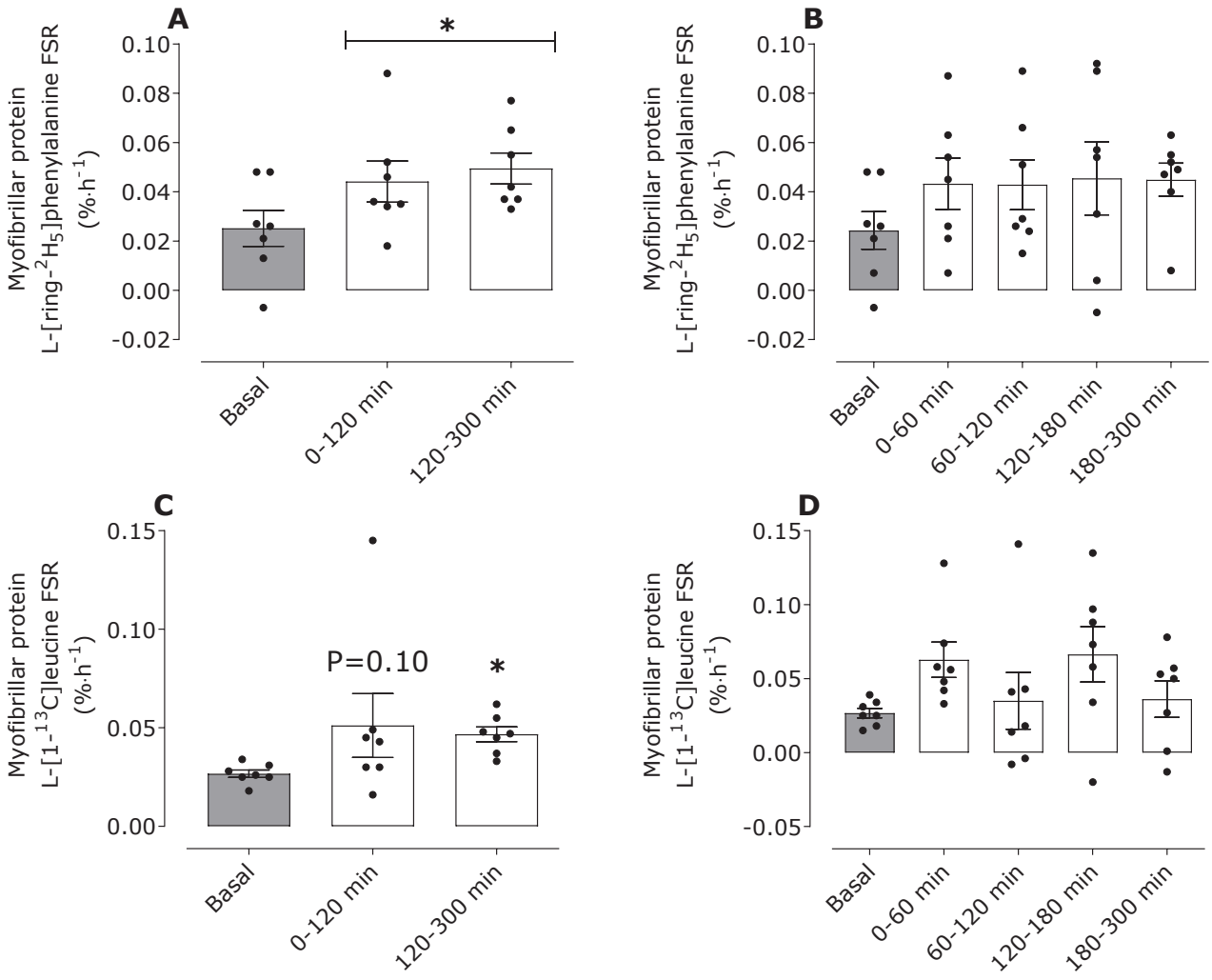

FIGURE 5

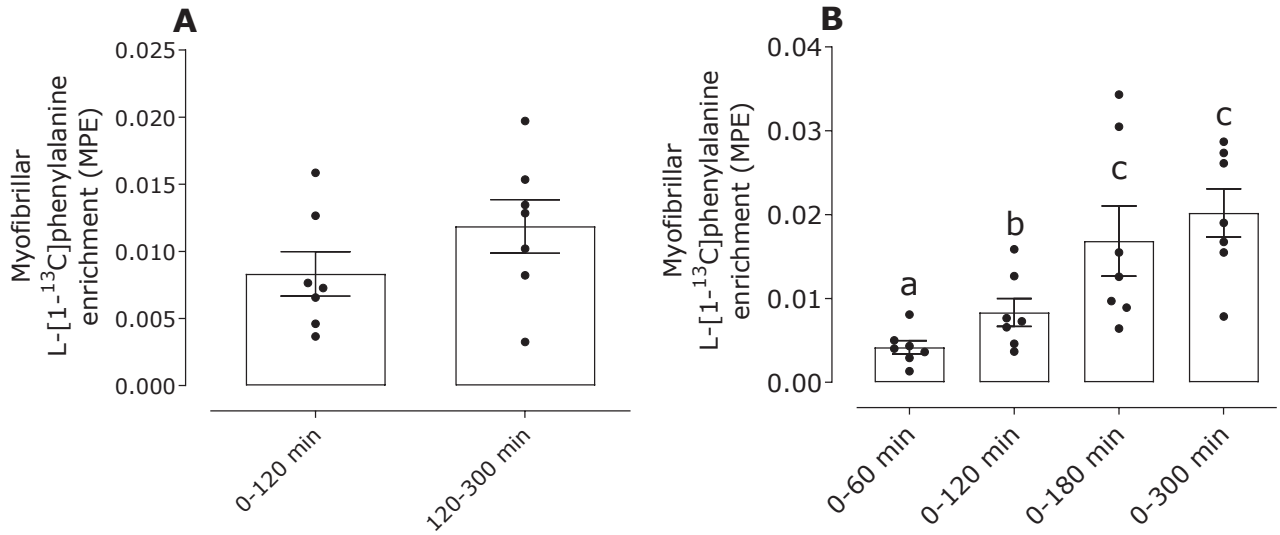

TABLE 1. Participants' characteristics (*n*=7).

Variable			
Age (y)	22	±	1.3
Weight (kg)	79.2	±	4.9
BMI (kg/m^2)	24.8	±	1.2
Systolic BP (mmHg)	124.7	±	3.9
Diastolic BP (mmHg)	72.4	±	3.9
Fat (%)	16.6	±	1.4
Lean Body Mass (kg)	64.0	±	3.7
Appendicular Lean Mass (kg)	29.0	±	1.7
Fasting Glucose (mg/dL)	78.3	±	1.0
Energy Intake (MJ/d)	8.74	±	0.4
Protein Intake (g/d)	113.0	±	4.8
Carbohydrate Intake (g/d)	228.7	±	21.1
Fat Intake (g/d)	83.0	±	1.17

Data are mean ± SEMs.

TABLE 2. Plasm amino acid, glucose, and insulin concentrations in the basal state and after ingestion of milk protein (38 g) in healthy young men (n=7).

	Time after drink (min)							
	0	30	60	90	120	180	240	300
Phenylalanine (μm)	60 ± 3	97 ± 5*	83 ± 3*	79 ± 4*	76 ± 3*	70 ± 3*	70 ± 4*	69 ± 4*
Tyrosine (μm)	55 ± 2	116 ± 10*	99 ± 7*	93 ± 5*	90 ± 5*	80 ± 4*	79 ± 4*	74 ± 3*
Leucine (μm)	130 ± 4	335 ± 32*	273 ± 24*	245 ± 15*	220 ± 8*	195 ± 8*	210 ± 9*	198 ± 11*
Glucose ($mg \cdot dL^{-1}$)	75 ± 1	81 ± 1*	77 ± 2	77 ± 1	76 ± 1	75 ± 1	75 ± 1	75 ± 2
Insulin ($\mu IU \cdot mL^{-1}$)	4 ± 1	19 ± 4*	9 ± 3	8 ± 3	6 ± 2	4 ± 1	–	3 ± 1

Data are mean ± SEMs. Data were analyzed with one-way repeated-measures ANOVA (time). The LSD test was used to locate differences between means when significance was observed. All time effect, P<0.01. *Different from basal (t=0 min).

TABLE 3. The change in myofibrillar protein bound labeling between two muscle biopsies in the basal state and after ingestion of milk protein (38 g) in healthy young men (*n*=7).

	Basal	Postprandial			
Tracer	−120-0 min	0-60 min	60-120 min	120-180 min	180-300 min
Δ Muscle Protein Bound, MPE					
L-[1-^{13}C]phenylalanine	–	0.0042 ± 0.0008	0.0041 ± 0.0014	0.0085 ± 0.0027	0.0033 ± 0.0026
L-[*ring*-$^{2}H_5$]phenylalanine	0.0036 ± 0.0010	0.0025 ± 0.0006	0.0023 ± 0.0005	0.0028 ± 0.0009	0.0058 ± 0.0009
L-[1-^{13}C]leucine	0.0036 ± 0.0003	0.0053 ± 0.0014	0.0032 ± 0.0016	0.0059 ± 0.0016	0.0062 ± 0.0021

Data are mean ± SEMs. MPE, mole percent excess.