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A single session of neuromuscular electrical stimulation does not augment postprandial muscle protein accretion

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Abstract

Background: The loss of muscle mass and strength that occurs with aging, termed sarcopenia, has been (at least partly) attributed to an impaired muscle protein synthetic response to food intake. We previously showed that neuromuscular electrical stimulation (NMES) can stimulate fasting muscle protein synthesis rates and prevent muscle atrophy during disuse. We hypothesized that NMES prior to protein ingestion would increase postprandial muscle protein accretion.

Methods: Eighteen healthy, elderly (69±1 y) males participated in this study. After performing a 70 min unilateral NMES protocol, subjects ingested 20 g intrinsically L-[1-^{13}C]-phenylalanine-labeled casein. Plasma samples and muscle biopsies were collected to assess postprandial mixed muscle and myofibrillar protein accretion, as well as associated myocellular signaling, during a 4 hour postprandial period in both the control (CON) and stimulated (NMES) leg.

Results: Protein ingestion resulted in rapid increases in both plasma phenylalanine concentrations and L-[1-^{13}C]-phenylalanine enrichments, which remained elevated during the entire 4 h postprandial period (P<0.05). Mixed muscle protein bound L-[1-^{13}C]-phenylalanine enrichments significantly increased over time following protein ingestion, with no differences between the CON (0.0164±0.0019 MPE) and NMES (0.0164±0.0019 MPE) leg (P>0.05). In agreement, no differences were observed in the postprandial rise in myofibrillar protein bound L-[1-^{13}C]-phenylalanine enrichments between the CON and NMES legs (0.0115±0.0014 vs 0.0133±0.0013 MPE, respectively; P>0.05). Significant increases in mTOR and P70S6K phosphorylation status were observed in the NMES stimulated leg only (P<0.05).

Conclusion: A single session of NMES prior to food intake does not augment postprandial muscle protein accretion in healthy, older men.

Abstract word count: 247

Introduction

Aging is accompanied by declines in skeletal muscle mass and strength, termed sarcopenia (24). A less than optimal diet and sedentary lifestyle are factors contributing to sarcopenia (24, 30). However, the underlying mechanisms remain to be elucidated. From a physiological perspective, any loss of muscle mass must be attributed to an imbalance between muscle protein synthesis and breakdown rates. Research has generally demonstrated that basal (i.e. postabsorptive) muscle protein synthesis (8, 18, 36, 41) and breakdown (37, 48) rates do not change with advancing age. As such, research has since focused on the impact of aging on the anabolic response to food intake. Recent work has shown that the skeletal muscle protein synthetic response to dietary protein ingestion is impaired in older individuals (8, 20, 41). This 'anabolic resistance' to food intake is now regarded as a key factor in the etiology of sarcopenia (26, 41). Accordingly, we (18, 23, 42) and many others (8, 25, 29, 32) have begun to investigate ways to overcome anabolic resistance in older individuals in an effort to develop more effective strategies to attenuate age-related muscle loss and support healthy aging.

One strategy that has been shown to be effective to increase the postprandial muscle protein synthetic response to feeding is physical activity performed prior to food ingestion (6, 28, 34, 49, 50). By combining the ingestion of a meal-like bolus (i.e. 20 g) of intrinsically-labelled milk protein with the continuous infusion of stable isotope-labelled amino acids, we were able to show that a single bout of physical activity performed prior to protein ingestion increases postprandial muscle protein synthesis rates, with more of the dietary protein derived amino acids being used as precursors for *de novo* muscle protein accretion (28). However, some conditions do not allow an increase in physical activity level. For example, acute periods of illness or injury necessitate short periods of bed rest or limb immobilization. Such successive short periods of local or whole-body muscle disuse increase anabolic resistance to feeding and contribute to the development of sarcopenia during the lifespan (21, 39). Therefore, alternative strategies to maximize the postprandial muscle protein synthetic response to food ingestion are warranted in both health and disease.

In situations where physical activity levels are reduced, neuromuscular electrical stimulation (NMES) may be used as an alternative means to elicit muscle contraction. We have previously shown that NMES increases (fasting) muscle protein synthesis rates (40), and can be applied effectively to prevent muscle atrophy during short periods of muscle disuse in young men (11) as well as critically ill patients (9). In the present study, we hypothesized that a single bout of NMES improves postprandial protein accretion by increasing the postprandial use of dietary protein derived amino acids for *de novo* muscle protein synthesis in older adults. To test this hypothesis, we selected 18 healthy older males who were subjected to 70 min of unilateral NMES followed by the ingestion of 20 g intrinsically L-[1-^{13}C]-phenylalanine-labelled casein protein. This was combined with regular blood and muscle tissue sampling to assess postprandial protein accretion and underlying myocellular signaling in both the stimulated and non-stimulated leg.

Methods

Subjects

Eighteen healthy, elderly men (age 69±1 y) were selected to participate in the present study. Subjects were excluded if one of the following criteria were met: BMI below 18.5 or above 30 $kg \cdot m^{-2}$, type 2 diabetes mellitus, use of non-steroidal anti-inflammatory drugs, presence of a pacemaker or implantable cardioverter defibrillator, or having participated in any regular resistance-type exercise program within 6 months prior to the study. Subjects' characteristics are displayed in **Table 1**. All subjects were informed on the nature and risks of the study before written informed consent was obtained. The study was approved by the Medical Ethical Committee of the Maastricht University Medical Centre^{+} in accordance with the Declaration of Helsinki.

Pretesting

All subjects filled out a health questionnaire and completed a routine medical screening before inclusion into the study. During this visit, an Oral Glucose Tolerance Test (OGTT) was performed in a fasted state (2) to test for type 2 diabetes mellitus, and height and weight were measured. A second visit was performed to assess body composition via whole-body dual energy x-ray absorptiometry (DEXA) and single-slice computed tomography (CT) of *m. quadriceps femoris*, at 15 cm above the patella. Also during this visit, subjects were familiarized with the NMES protocol to be used in the experimental visit (see below for details).

Diet and physical activity prior to testing

The evening prior to the test day, subjects consumed a standardized meal containing 2900 kJ providing 51 energy% (en%) as carbohydrate, 32 en% as fat, and 17 en% as protein. All subjects received instructions to refrain from any sort of heavy physical activity and to keep their diet as constant as possible during the 48 h prior to the test day.

Experimental protocol

An overview of the experimental protocol is depicted in **Figure 1**. After an overnight fast, subjects arrived at the laboratory at 8:00 AM for a single test day. While resting in a supine position on a bed, a catheter was placed in a heated dorsal hand vein and placed in a hot box at 60ºC for arterialized venous blood sampling (1). After collection of a basal arterialized blood sample at t = -210 min, a blood sample was collected 120 min (t = -90 min) after the baseline sample. After this, an NMES protocol (see below for details) was started at t = -70 min. After terminating the NMES session at t = 0 min, a blood sample was taken, and muscle biopsies were collected from both the stimulated (NMES) and the non-stimulated (CON) leg within approximately 5 min after the end of the NMES protocol. Subjects then received a test drink containing 20 g intrinsically L-[1-^{13}C]-phenylalanine-labeled protein. The consumption of this drink signified the beginning of a 4 h postprandial period. Arterialized blood samples were subsequently collected every 60 min with the final sample being taken at t = 240 min. At the same time (at t = 240 min), muscle biopsy samples were taken from both the NMES and CON leg.

Arterialized venous blood samples were collected into pre-cooled EDTA-containing tubes and centrifuged at 1000*g* for 10 min at 4ºC. Aliquots of plasma were snap frozen in liquid nitrogen and stored at -80ºC until further analysis. Muscle biopsy samples were collected from the middle region of *m. vastus lateralis*, ~15 cm above the patella (4). Any visible non-muscle tissue was removed, and the muscle sample was frozen in liquid nitrogen. Subsequently, samples were stored at -80ºC until further analysis.

Neuromuscular electrical stimulation

After inclusion, one of the subjects' legs was randomly allocated to receive 70 min of NMES during the experimental visit. Prior to the NMES session, subjects were placed in a supine position with a pillow underneath both knees to instigate light knee flexion. Four self-adhesive electrodes (50 x 50 mm; Enraf-Nonius, Rotterdam, the Netherlands) were placed on the distal part at the muscle belly of the *m. rectus femoris* and the *m. vastus lateralis*, and at the inguinal area of both muscles of both legs. The electrodes

were connected to an Enraf-Nonius TensMed S84 stimulation device, discharging biphasic symmetric rectangular-wave pulses. However, NMES was only applied to one leg (NMES) while the other leg served as a sham-treated control (CON). The 70-min protocol consisted of a warm-up phase (5 min, 5 Hz, 250 μs), a stimulation period (60 min, 100 Hz, 400 μs, 5 s on (0.75 s rise, 3.5 s contraction, 0.75 s fall) and 10 s off), and a cooling-down phase (5 min, 5 Hz, 250 μs). This protocol was selected as we previously demonstrated it is effective in preventing muscle atrophy during short-term disuse in young men (11) and critically ill patients (9). Subjects were encouraged to continuously adjust the intensity of the stimulation to the level where a full contraction of *m. quadriceps femoris* was both visible and palpable, with the heel slightly being lifted from the bed. The NMES protocol was completed by all subjects. The maximal intensity of the 70 min NMES session averaged 35.9±2.7 mA, whereas the average intensity across all subjects and sessions averaged 26.1±1.5 mA.

Preparation of intrinsically labeled protein

Intrinsically L-[1-^{13}C]-phenylalanine-labeled micellar casein protein was obtained by infusing a Holstein cow with large quantities of L-[1-^{13}C]-phenylalanine, collecting milk, and purifying the casein fraction as described previously (35). The average L-[1-^{13}C]-phenylalanine enrichment was 38.7 mole percent excess (MPE). All subjects received a drink with 20 g casein in a total volume of 350 mL, flavored with vanilla flavor.

Plasma analyses

Plasma glucose and insulin concentrations were analyzed by Dr. Stein und Kollegen Laboratories (Mönchengladbach, Germany) using commercially available kits (GLUC3, Roche, Ref: 05168791 190, and Immunologic, Roche, Ref: 12017547 122, respectively). Plasma amino acid concentrations and enrichments were determined by GC-MS (Agilent 7890A GC/5975C; MSD, Little Falls, DE, USA). Plasma phenylalanine was converted to its tert-butyl dimethylsilyl (TBDMS) derivative before analysis by GC-MS by using electron impact ionization by monitoring ions at mass/charge (*m/z*) 336 and 337 for

160 unlabeled and [1-^{13}C]-labeled phenylalanine, respectively (42). Standard regression curves were applied
161 from a series of known standard enrichment values against the measured values to assess the linearity of
162 the mass spectrometer and to account for any isotope fractionation which may have occurred during the
163 analysis. Phenylalanine enrichments were corrected for the presence of the ^{13}C isotopes.
164
165 *Muscle tissue analyses*
166 Mixed muscle protein bound enrichments were determined in a piece of wet muscle (~45 mg) as
167 described previously (18). Briefly, muscle was freeze-dried, and collagen, blood and other visible non-
168 muscle material was removed under a dissecting microscope. After homogenizing and incubating samples
169 in ice-cold 2% perchloric acid (PCA), samples were centrifuged. The supernatant was collected for
170 determination of L-[1-^{13}C]-phenylalanine enrichments in the muscle free amino acid pool using GC-MS
171 analysis (42). The mixed muscle protein pellet washed, hydrolyzed overnight, and dried under a nitrogen
172 stream. Next, free amino acids were dissolved in 50% acetic acid solution and passed over cation
173 exchange AG 50W0X8 resin columns. To determine the L-[1-^{13}C]-phenylalanine enrichment, the purified
174 amino acids were derivatized into their N(O,S)-ethoxycarbonyl ethyl ester derivatives with ethyl
175 chloroformate (ECF), and then measured by GC-C-IRMS (MAT 253; Thermo Scientific, Bremen,
176 Germany) using a DB5-MS-column (no. 122122-5532; Agilent J+W, USA), GC Isolink, and monitoring
177 ion masses 44, 45, and 46. By establishing the relation between the enrichment of a series of L-[1-^{13}C]-
178 phenylalanine standards of variable enrichments and the enrichments of the N(O,S)-ethoxycarbonyl ethyl
179 esters of these standards, the mixed muscle protein-bound enrichment of phenylalanine was determined.
180 Myofibrillar protein enriched fractions were extracted from wet muscle tissue as described elsewhere (5).
181 In short, ~50 mg wet muscle tissue was manually homogenized on ice using a Teflon pestle in a standard
182 extraction buffer, after which the samples were centrifuged and the supernatants containing sarcoplasmic
183 proteins were removed. In an additional step, the myofibrillar fraction-containing supernatant was
184 collected and the collagen pellet was removed. The remaining myofibrillar fraction was purified and
185 hydrolyzed, such that the free amino acids remained and could be dried under a nitrogen stream. The

186 enrichment of the derivative was measured by GC-C-IRMS by using a DB5-MS-column (no. 122-5532;
187 Agilent J+W, USA), GC Isolink, and monitoring of ion masses 44, 45, and 46. By establishing the
188 relationship between the enrichment of a series of L-[1-^{13}C]-phenylalanine standards of variable
189 enrichment, the myofibrillar protein-bound enrichment of phenylalanine was determined. Standard
190 regression curves were applied to assess the linearity of the mass spectrometer and to control for the loss
191 of tracer. Muscle protein deposition from the ingested casein over the 4 h postprandial period was
192 expressed as the relative increase of L-[1-^{13}C]-phenylalanine enrichment in muscle tissue.
193 Western blot analyses were performed as described previously (9). In short, ~30 mg muscle tissue was
194 homogenized and protein quantification was performed. After protein quantification, the gels were
195 transferred onto a nitrocellulose membrane. Specific proteins were detected by overnight incubation with
196 the following antibodies: anti-mTOR (289 kDa; dilution 1:1000, #2972 Cell Signaling, Danvers, MA,
197 USA) and anti-phospho-mTOR (Ser^{2448}; 289 kDa, dilution 1:1000, #2971 Cell Signaling), anti-P70S6K
198 (70 kDa; dilution 1:1000, #9202 Cell Signaling) and anti-phospho P70S6K (Thr^{389}; 70 kDa, dilution
199 1:1000, #9206 Cell Signaling), anti-RS6 (32 kDa; dilution 1:1000; #2217 Cell Signaling) and anti-
200 phospho-RS6 (Ser^{235}/Ser^{236}, 32 kDa; dilution 1:1000; #4856 Cell Signaling) and anti α-tubulin (52 kDa;
201 dilution 1:1000; #2125 Cell Signaling). The complementary secondary antibodies applied were IRDye
202 680 donkey anti-rabbit (Cat. No. 926-32223, dilution 1:10000, Li-Cor, Lincoln, NE, USA) and IRDye
203 800CW donkey anti-mouse (Cat. No. 926-32212, dilution 1:10000, Li-Cor). Protein quantification was
204 performed by scanning on an Odyssey Infrared Imaging System (LI-COR Biotechnology, Lincoln, NE,
205 USA).
206
207 *Statistics*
208 All data are expressed as means±SEM. Differences in baseline leg values (i.e. left vs right) were
209 determined using a paired samples t-test. A one-way repeated measures analysis of variance (ANOVA)
210 with time as within-subjects factor was used to analyze effects in plasma concentrations and enrichments.
211 Differences in protein-bound L-[1-^{13}C]-phenylalanine enrichments between legs after 4 h incorporation

were analyzed using a paired-samples t-test. When a significant main effect was detected, Bonferroni's post hoc test was applied to locate the differences. Statistical analyses were performed using the SPSS version 22.0 software package (SPSS Inc., Chicago, IL, USA), with statistical significance set at $P<0.05$.

Results

Plasma analyses

For plasma glucose and insulin concentrations, depicted in **Figure 2**, a significant time effect was observed (both $P<0.001$). During the postprandial period, plasma glucose and insulin concentrations averaged 5.6±0.1 mmol·L^{-1} and 7.5±0.8 mU·L^{-1}, respectively. **Figure 3** displays plasma concentrations of phenylalanine (**A**), tyrosine (**B**), and leucine (**C**). At the start of the experiment, fasting plasma phenylalanine, tyrosine, and leucine concentrations averaged 54±1, 62±2 and 128±5 μM, respectively. Following the ingestion of 20 g casein, at t = 0 min, concentrations of these three amino acids increased rapidly (time effect; $P<0.001$) and remained elevated until the end of the experiment. **Figure 4** depicts plasma enrichments of L-[1-^{13}C]-phenylalanine. After ingestion of the protein beverage, plasma L-[1-^{13}C]-phenylalanine enrichments increased ($P<0.001$), and remained elevated throughout the 4 h postprandial period.

Muscle tracer analyses

Muscle free L-[1-^{13}C]-phenylalanine enrichments averaged 4.1±0.2 and 4.1±0.2 MPE at 4 h after protein ingestion in the CON and NMES leg, respectively, which did not differ (P>0.05). **Figure 5A** depicts mixed muscle L-[1-^{13}C]-phenylalanine enrichments following ingestion of 20 g casein in the CON and NMES leg. Four hours after the ingestion of 20 g casein protein, mixed muscle L-[1-^{13}C]-phenylalanine enrichments did not differ between legs: 0.0164±0.0019 and 0.0164±0.0019 MPE in the CON and NMES leg, respectively ($P>0.05$). L-[1-^{13}C]-phenylalanine enrichments of the intracellular free amino acid pool were 4.074±0.183 and 4.115±0.163 MPE in the CON and NMES leg, respectively ($P>0.05$).

Myofibrillar protein bound L-[1-^{13}C]-phenylalanine enrichments are presented in **Figure 5B**. Ingestion of 20 g casein resulted in an increase in L-[1-^{13}C]-phenylalanine enrichments up to 0.0115±0.0014 and 0.0133±0.0013 MPE in the CON and NMES leg, respectively ($P>0.05$).

241 *Signaling proteins*

242 The muscle phosphorylation status of selected proteins involved in the regulation of muscle protein
243 synthesis is displayed in **Figure 6**. Data are expressed as the ratios between the phosphorylated protein
244 and the total protein content. Directly after cessation of the NMES, for P70S6K, a higher phosphorylation
245 status was observed in the NMES leg when compared to the CON leg ($P<0.05$, **Figure 6B**). Following
246 protein ingestion, the phosphorylation status of mTOR (**Figure 6A**) significantly increased over time in
247 the NMES leg only (interaction effect; $P<0.05$). No changes in the phosphorylation status of P70S6K
248 were observed after protein ingestion between legs or over time. Despite a significant interaction effect
249 for RS6 (**Figure 6C**; $P<0.01$), no changes over time were found in the CON and NMES legs.

Discussion

In the present study we show that neuromuscular electrical stimulation (NMES) prior to protein ingestion does not augment the use of dietary protein derived amino acids for *de novo* muscle protein accretion in healthy, older males. Nevertheless, we observed significant increases in mTOR and P70S6K phosphorylation in muscle following the bout of NMES.

Aging is accompanied by declines in skeletal muscle mass and strength, called sarcopenia (24). Previous research has shown that the older population possesses a blunted skeletal muscle protein synthetic response to food intake, termed 'anabolic resistance' (8, 20, 41). This anabolic resistance is now believed to represent a key factor in the etiology of sarcopenia (26, 41). In the current study, intake of a meal-like amount of 20 g intrinsically-labeled casein led to a rapid increase in both plasma insulin (**Figure 2**) and amino acid concentrations (**Figure 3**), which was accompanied by an increase in plasma L-[1-^{13}C]-phenylalanine enrichment that remained elevated for the entire 4 h postprandial period (**Figure 4**). Taken together, all prerequisites were provided for an increase in anabolic signaling with ample amino acids made available as precursors to support postprandial muscle protein accretion. Indeed, these dietary protein derived amino acids were rapidly used for *de novo* muscle protein synthesis, as evidenced by the ~0.016 MPE increase in muscle protein bound L-[1-^{13}C]-phenylalanine in mixed muscle tissue and ~0.012 MPE in the myofibrillar fraction of the muscle tissue obtained in the control leg 4 h after protein ingestion (**Figure 5**). The use of intrinsically L-[1-^{13}C]-phenylalanine labeled protein allows us to assess the percentage of the ingested protein that was released into the circulation and used for *de novo* muscle protein synthesis (19). Based on the assumption that L-[1-^{13}C]-phenylalanine enrichments in *m. vastus lateralis* would be representative of most other muscle groups, we calculated that a total of 0.037±0.004 g L-[1-^{13}C]-phenylalanine had been incorporated in all appendicular lean tissue during the entire 4 h postprandial period. This translates to 2.0±0.2 g muscle protein, and equals 9.9±1.2% of the ingested dietary protein derived amino acids that were incorporated in *de novo* muscle protein. These data are in line with our recent calculations (19) and demonstrate the possibilities of using intrinsically labeled protein to demonstrate the metabolic fate of dietary protein derived amino acid *in vivo* in humans (35).

Physical activity performed prior to food intake has been shown to further increase postprandial muscle protein synthesis compared with food intake alone (6, 28, 34, 49, 50), and to augment the use of protein derived amino acids for *de novo* muscle protein synthesis (28). Currently, it remains unknown to what extent the stimulating properties of physical activity are attributed to its impact on skeletal muscle perfusion or whether the effects are predominantly intramuscular. As maintaining or increasing physical activity can be compromised in various clinical and non-clinical settings, exercise mimetics such as NMES may be used to evoke involuntary contractions to reintroduce some level of physical activity. Indeed, previous work from our group has shown that local NMES can increase post-absorptive muscle protein synthesis rates by as much as 27% when compared to the non-stimulated, control leg (40). To date, no studies have assessed the impact of NMES on the postprandial muscle protein synthetic response to feeding. In the current study, we assessed postprandial protein accretion following ingestion of a single bolus of intrinsically labelled protein in an electrically stimulated (NMES) and a non-stimulated, control leg. Despite the 70 min of neuromuscular electrical stimulation prior to protein ingestion we observed no differences in the muscle free [1-^{13}C]-phenylalanine enrichments or the deposition of dietary protein derived amino acids into *de novo* muscle protein between both legs (0.0164±0.0019 vs 0.0164±0.0019 MPE and 0.0115±0.0014 vs 0.0133±0.0013 MPE for the increase in [1-^{13}C]-phenylalanine enrichment in mixed muscle protein and myofibrillar protein, respectively; **Figure 5**). Clearly, a single session of NMES prior to protein ingestion was not sufficient to modulate the metabolic fate of the dietary protein derived amino acids and did not augment postprandial protein deposition in the stimulated leg of these healthy, older males. In the current study, we employed a within-subjects design to eliminate between-subject variability and isolate the local impact of NMES on muscle protein synthesis. It could be suggested that NMES may exert systemic effects that stimulate muscle protein synthesis in both the stimulated as well as the control leg (31, 51). However, previous work (46, 47) as well as the lack of differences in anabolic signaling between the CON and NMES leg (**Figure 6**) provide little evidence for such a 'spillover' effect during the early stages of recovery from NMES.

301 The postprandial stimulation of muscle protein synthesis is initiated by a phosphorylation cascade in
302 which mammalian target of rapamycin (mTOR) and its downstream effectors P70S6 kinase (P70S6K)
303 and ribosomal protein S6 (RS6) are key players (14, 22). This pathway is not only activated by protein
304 intake, but also by physical activity (as reviewed in (45)). Here we show that protein ingestion did not
305 lead to changes in activation of mTOR, P70S6K, and RS6 in the control leg (**Figure 6**). This is not
306 surprising considering our low dose of protein administered (27) as well as previous work showing the
307 peak of this translation initiation process to generally occur 1-2 h following protein ingestion, and to
308 subside thereafter (7, 12, 17). Of course, the timing of our muscle biopsy collection that was chosen to
309 optimally measure muscle protein-bound enrichments was likely not optimal for the detection of changes
310 in anabolic signaling, which had probably subsided by then. However, we observed an early increase in
311 the phosphorylation of P70S6K immediately following NMES (**Figure 6**). This is in agreement with our
312 previous work demonstrating that an acute bout of NMES stimulates muscle protein synthesis in the
313 postabsorptive state, possibly via a similar rise in P70S6K signaling (40), but is rather contradictory to
314 previous studies showing an increase in P70S6K to occur only several hours after the cessation of
315 exercise (7, 17). Previously, we have observed increases in P70S6K phosphorylation during or
316 immediately after exercise without measurable increases in mTOR phosphorylation (3), which shows that
317 activation of these pathways can occur early after the onset of exercise or electrostimulation before a
318 measurable increase in mTOR activation. A significant increase in mTOR activation was not observed
319 until 4 h after NMES (**Figure 6**). This is in line with previous data showing greater mTOR
320 phosphorylation at 4 h after NMES (40) and 3 h postexercise (13) in older individuals. Clearly, the NMES
321 did induce an anabolic stimulus, but this did not seem strong enough to augment the postprandial muscle
322 protein synthetic response to feeding.
323 Previously, we have shown that muscle loss during disuse can be prevented by the application of NMES
324 in both young males during short-term immobilization (11) as well as in critically ill patients in a
325 comatose state (9). This has been, at least partly, attributed to the increase in basal muscle protein
326 synthesis rate that can be observed after performing a single session of NMES (40). In the present study,

we assessed whether NMES augmenting the muscle protein synthetic response to feeding may go some way to explaining the beneficial effect on muscle retention during disuse. In contrast to our hypothesis, we failed to detect a stimulatory effect of NMES on postprandial muscle protein accretion. This implies that NMES may particularly impact upon basal protein synthesis rates, as opposed to postprandial protein handling, in healthy older men. Although muscle disuse is associated with anabolic resistance to food intake (17, 38, 43), based on the present data it could be suggested that the observed efficacy of NMES to prevent disuse atrophy is primarily attained in the basal state (15, 16, 38). Though our results demonstrate that NMES does not affect postprandial protein handling in healthy, active individuals, we cannot exclude that NMES may modulate postprandial protein handling in a more clinically compromised state, where anabolic sensitivity to food intake is further reduced (12, 17, 38, 43) or in fact under situations where larger amounts, or more anabolic dietary proteins are provided to older subjects. Obviously, the efficacy of NMES combined with nutritional support may be of particular relevance for older hospitalized patients, who are losing muscle partially due to low dietary protein intake (10, 33, 44). Furthermore, it should be noted that we assessed the effect of a single bout of NMES only, and we cannot rule out any synergistic effects of multiple, repetitive NMES sessions performed over time.

In conclusion, a single session of NMES prior to protein ingestion does not augment postprandial muscle protein accretion in healthy, older males.

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Author contributions

MLD, BTW, and LJCvL designed the study. MLD, BTW, and IFK organized and performed the experiments. AHZ, JG, and APG performed the muscle analyses. MLD analyzed the data. MLD, BTW, and LJCvL interpreted the data. MLD drafted the manuscript. MLD, BTW, and LJCvL edited and revised the manuscript. All authors approved the final version.

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506 exercise increases postprandial muscle protein synthesis in humans. *Medicine and science in sports and*
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512 arm exercise with eccentric and concentric muscle contractions in resistance-trained athletes with left
513 ventricular hypertrophy. *International journal of sports medicine* 34: 116-122, 2013.

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516

517 **Tables**

518

519 **Table 1**: Subjects' characteristics

Age (y)	69 ± 1
Weight (kg)	83.4 ± 2.2
BMI ($kg \cdot m^{-2}$)	26.5 ± 0.6
Body fat (% body weight)	22.0 ± 1.0
Lean body mass (kg)	62.5 ± 1.5
Leg lean mass (kg)	10.0 ± 0.3
Quadriceps CSA (mm^2)*	7151 ± 266
Leg volume (L)	8.4 ± 0.2
Basal plasma glucose ($mmol \cdot L^{-1}$)	5.7 ± 0.1
Basal plasma insulin ($mU \cdot L^{-1}$)	10.2 ± 1.0
HbA1c (%)	5.4 ± 0.1
OGIS ($mL \cdot min^{-1} \cdot m^{-2}$)	433 ± 9

520 Values represent means±SEM. BMI, body mass index; CSA, cross-sectional area; HbA1c, glycosylated
521 hemoglobin; OGIS, oral glucose insulin sensitivity. * Data from *n*=10 participants
522

Figure legends

Figure 1: Outline of the experimental protocol. Eighteen healthy, older men ingested a protein drink containing 20 g casein following unilateral NMES (neuromuscular electrical stimulation).

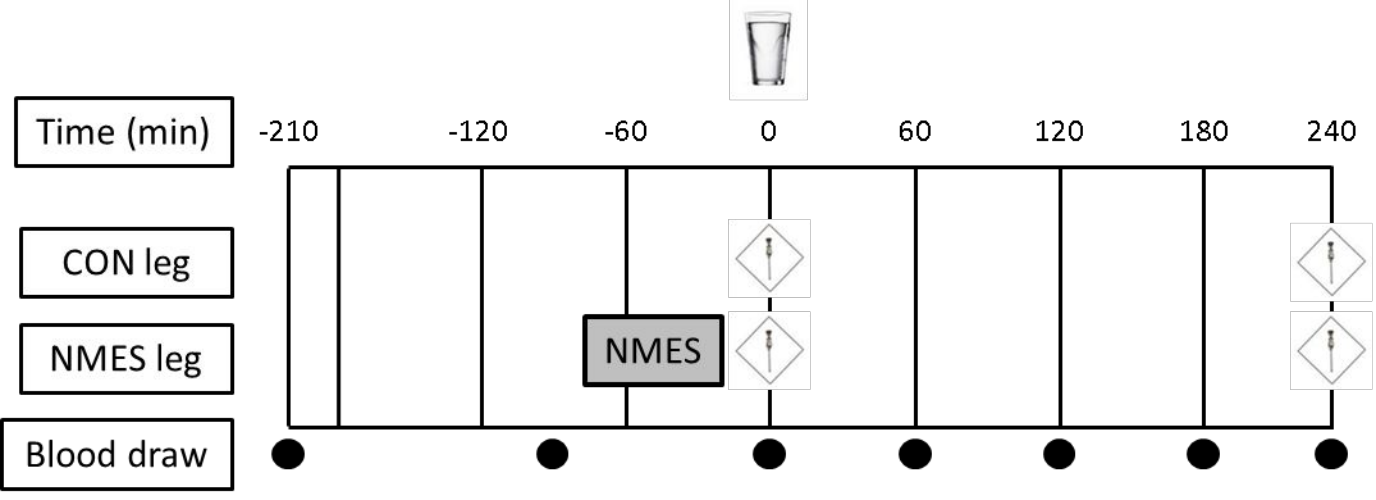
Figure 2: Means±SEM plasma glucose (**A**) and insulin (**B**) concentrations prior to and following ingestion of 20 g casein. The gray bar represents a 70 min NMES protocol. Data were analyzed with one-way repeated measures ANOVA with time as within-subjects factor. A significant time effect ($P<0.001$) was found for both glucose and insulin. * Significantly different from t = 0 min ($P<0.05$).

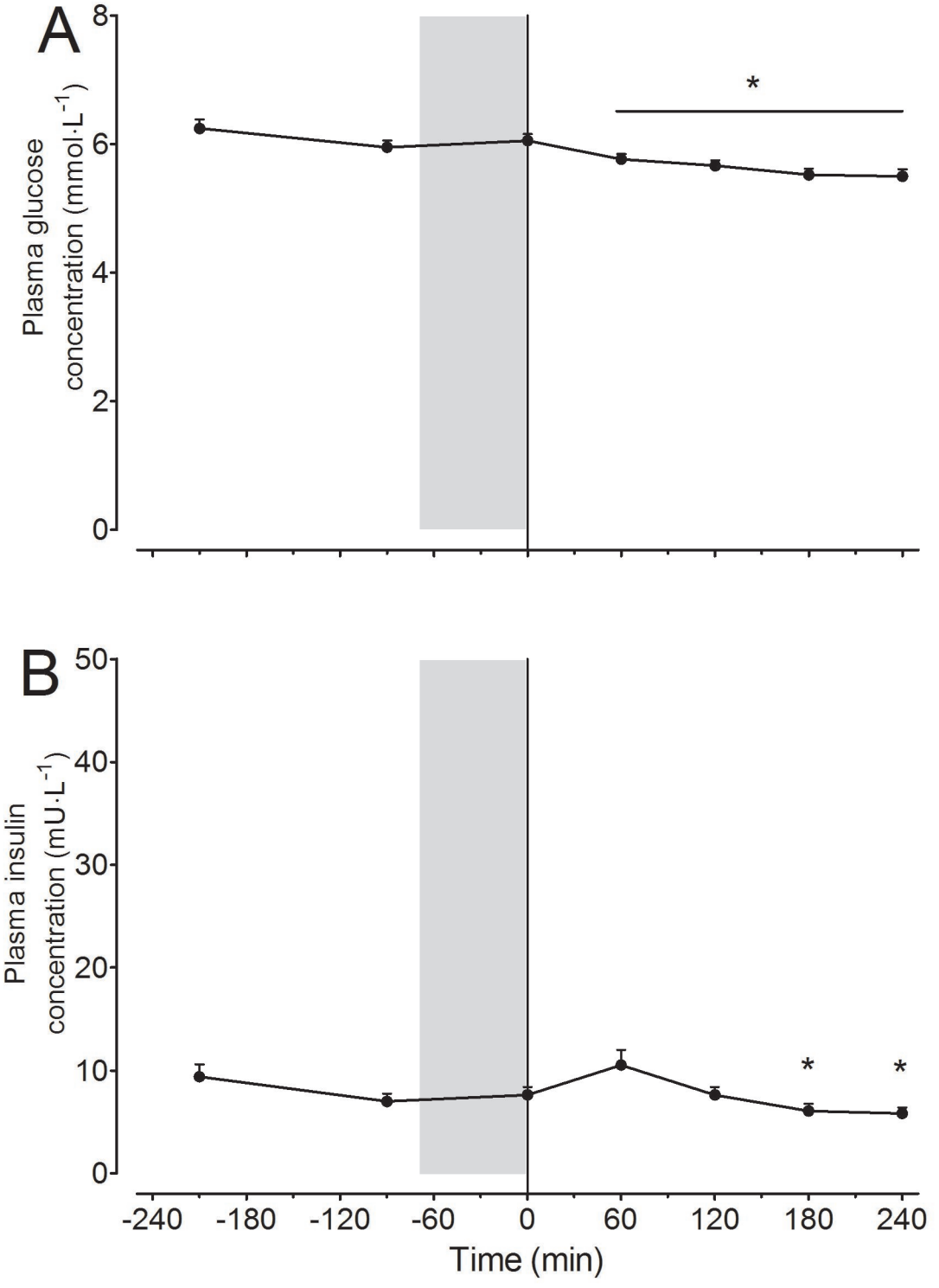
Figure 3: Mean±SEM plasma phenylalanine (**A**), tyrosine (**B**), and leucine (**C**) concentrations during the fasting period (t = -210 until 0 min) and following the ingestion of 20 g casein. The gray bar represents the 70 min NMES protocol. Data were analyzed with one-way repeated measures ANOVA with time as within-subjects factor. For all amino acids, significant time effects were observed (all $P<0.001$). * Significantly different from t = 0 min ($P<0.05$).

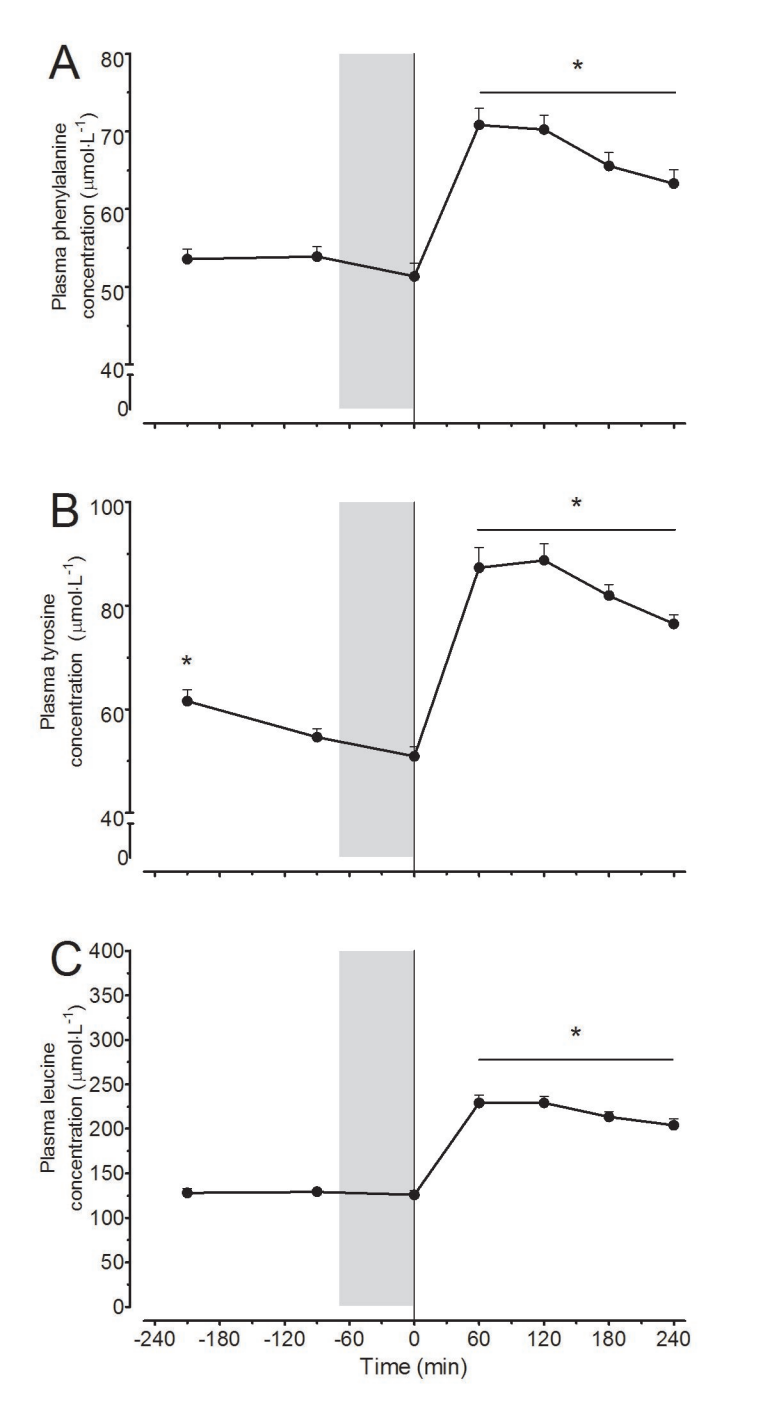
Figure 4: Plasma [1-^{13}C]-phenylalanine enrichments. The 70 min NMES protocol is visualized by the grey bar. Values are expressed as means±SEM. Data were analyzed with one-way repeated measures ANOVA with time as within-subjects factor. A significant time effect was found ($P<0.001$). * Significantly different from t = 0 min ($P<0.05$).

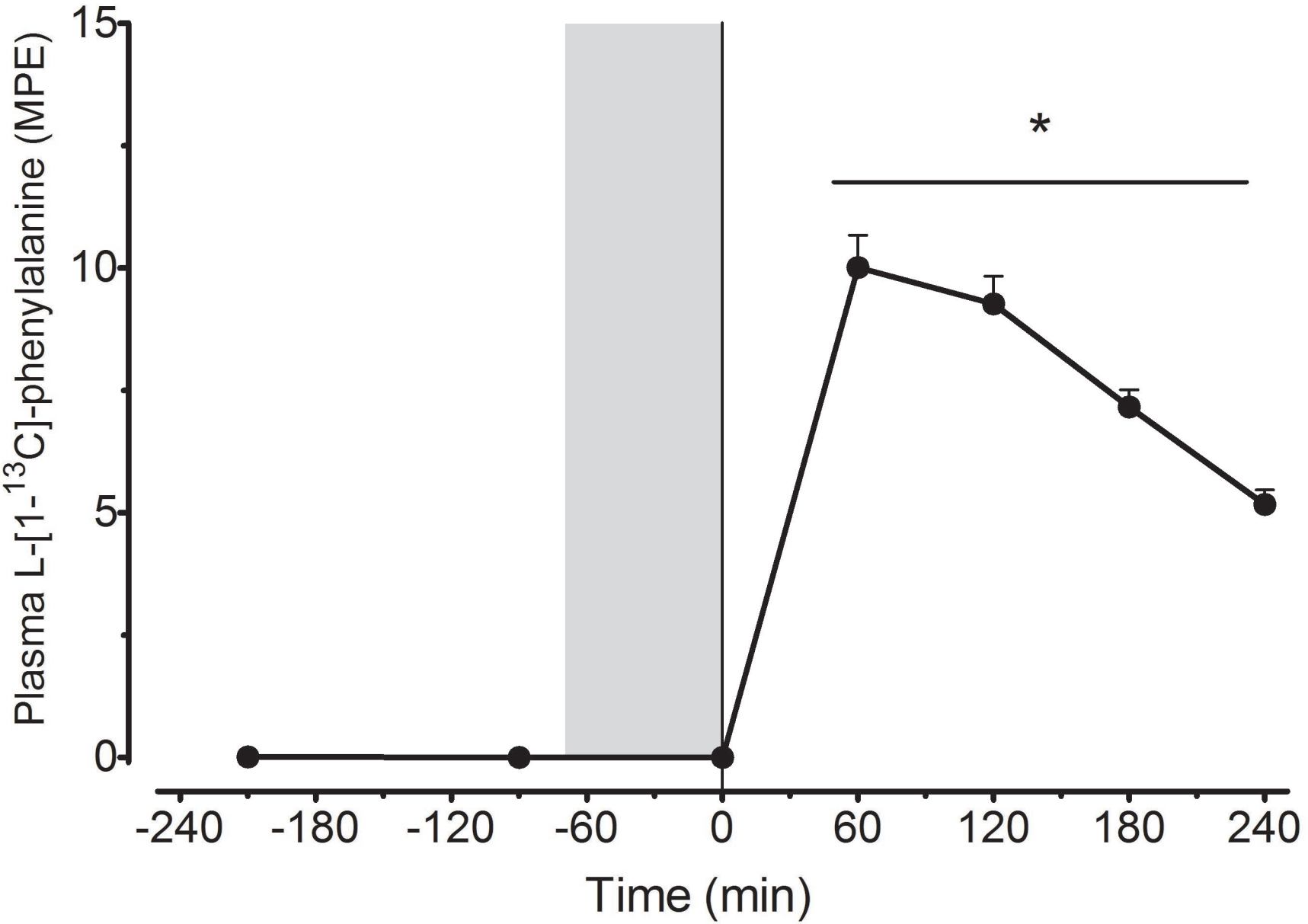
Figure 5: Individual subjects' mixed muscle (**A**) and myofibrillar protein-bound (**B**) L-[1-^{13}C]-phenylalanine enrichments (MPE) over a 4 h period following ingestion of 20 g casein, in the CON and NMES leg. Data are presented as means±SEM.

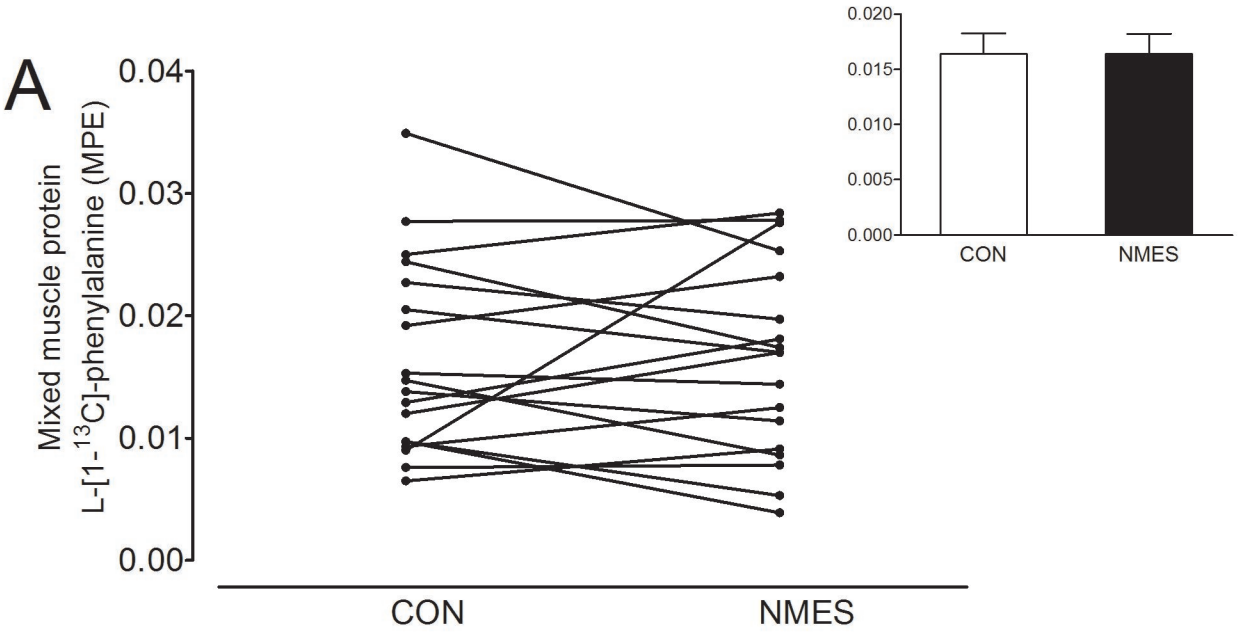
548 **Figure 6**: Skeletal muscle phosphorylation status (expressed as means±SEM) of selected proteins in the
549 control (CON) and stimulated (NMES) leg. Muscle samples were taken directly after (t = 0 min) ingestion
550 of 20 g casein protein, and 4 h thereafter (t = 240 min). * Significantly different from t = 0 min. #
551 Significantly different from CON. Abbreviations: mTOR, mammalian target of rapamycin; P70S6K,
552 P70S6 kinase; RS6, ribosomal protein S6.
553

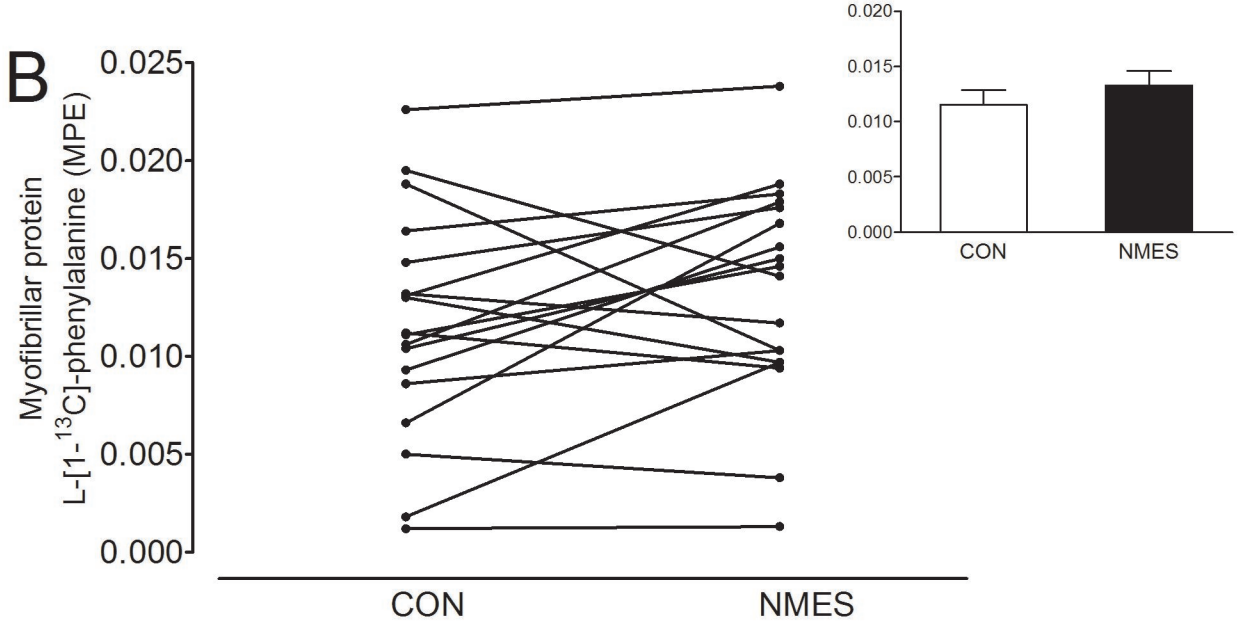

Time (min)
-210
-120
-60
0
60
120
180
240
CON leg
NMES leg
NMES
Blood draw

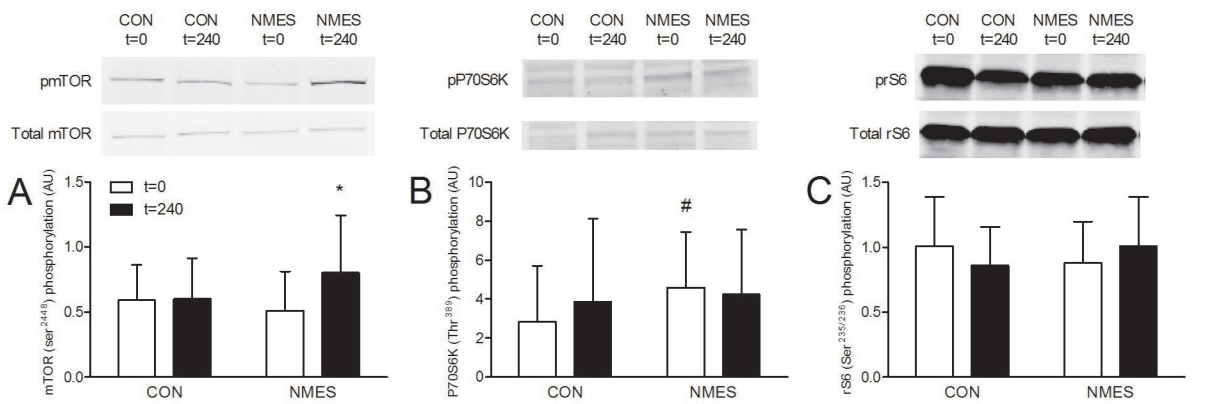

A
Plasma glucose concentration (mmol·L-1)
8
6
4
2
0
*
B
Plasma insulin concentration (mU·L-1)
50
40
30
20
10
0
*
*
-240
-180
-120
-60
0
60
120
180
240
Time (min)


A
Plasma phenylalanine concentration (μmol·L-1)
80
70
60
50
40
0
*
B
Plasma tyrosine concentration (μmol·L-1)
100
80
60
40
0
*
*
C
Plasma leucine concentration (μmol·L-1)
400
350
300
250
200
150
100
50
0
*
-240
-180
-120
-60
0
60
120
180
240
Time (min)


15
10
5
0
Plasma L-[1-13C]-phenylalanine (MPE)
*
-240
-180
-120
-60
0
60
120
180
240
Time (min)


A
Mixed muscle protein
L-[1-13C]-phenylalanine (MPE)
0.04
0.03
0.02
0.01
0.00
CON
NMES
0.020
0.015
0.010
0.005
0.000
CON
NMES


B
Myofibrillar protein
L-[1-13C]-phenylalanine (MPE)
0.025
0.020
0.015
0.010
0.005
0.000
CON
NMES
0.020
0.015
0.010
0.005
0.000
CON
NMES


CON t=0
CON t=240
NMES t=0
NMES t=240
pmTOR
Total mTOR
pP70S6K
Total P70S6K
prS6
Total rS6
A
B
C
t=0
t=240
*
#
mTOR (ser2448) phosphorylation (AU)
P70S6K (Thr389) phosphorylation (AU)
rS6 (Ser235/236) phosphorylation (AU)
CON
NMES