Short-term muscle disuse lowers myofibrillar protein synthesis rates and induces anabolic resistance to protein ingestion

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1Top Institute Food and Nutrition, Wageningen, The Netherlands; 2NUTRIM School for Nutrition, Toxicology, and Metabolism, Maastricht University Medical Centre, Maastricht, The Netherlands; and 3Department of Surgery, Maastricht University Medical Centre, Maastricht, The Netherlands

Submitted 14 May 2015; accepted in final form 11 November 2015

Wall BT, Dirks ML, Snijders T, van Dijk J, Fritsch M, Verdijk LB, van Loon LJ. Short-term muscle disuse lowers myofibrillar protein synthesis rates and induces anabolic resistance to protein ingestion. Am J Physiol Endocrinol Metab 310: E137–E147, 2016. First published November 17, 2015; doi:10.1152/ajpendo.00227.2015.—Disuse leads to rapid loss of skeletal muscle mass and function. It has been hypothesized that short successive periods of muscle disuse throughout the lifespan play an important role in the development of sarcopenia. The physiological mechanisms underlying short-term muscle disuse atrophy remain to be elucidated. We assessed the impact of 5 days of muscle disuse on postabsorptive and postprandial myofibrillar protein synthesis rates in humans. Twelve healthy young (22 ± 1 yr) men underwent a 5-day period of one-legged knee immobilization (full leg cast). Quadriceps cross-sectional area (CSA) of both legs was assessed before and after immobilization. Continuous infusions of L-[ring-2H5]phenylalanine and L-[1-13C]leucine were combined with the ingestion of a 25-g bolus of intrinsically L-[1-13C]phenylalanine- and L-[1-13C]leucine-labeled dietary protein to assess myofibrillar muscle protein fractional synthetic rates in the immobilized and nonimmobilized control leg. Immobilization led to a 3.9 ± 0.6%/h decrease in quadriceps muscle CSA of the immobilized leg. Based on the L-[ring-2H5]phenylalanine tracer, immobilization reduced postabsorptive myofibrillar protein synthesis rates by 41 ± 13% (0.015 ± 0.002 vs. 0.032 ± 0.005%/h; P < 0.01) and postprandial myofibrillar protein synthesis rates by 53 ± 4% (0.020 ± 0.002 vs. 0.044 ± 0.003%/h; P < 0.01). Comparable results were found using the L-[1-13C]leucine tracer. Following protein ingestion, myofibrillar protein bound L-[1-13C]phenylalanine enrichments were 53 ± 18%/h lower in the immobilized compared with the control leg (0.007 ± 0.002 and 0.015 ± 0.002 mole% excess, respectively, P < 0.05). We conclude that 5 days of muscle disuse substantially lowers postabsorptive myofibrillar protein synthesis rates and induces anabolic resistance to protein ingestion.

In otherwise healthy humans, the recovery from injury or illness often mandates a period of local (e.g., limb immobilization) or whole body (e.g., bed-rest) muscle disuse. It has long been recognized that a prolonged period of disuse (i.e., >10 days) leads to rapid skeletal muscle atrophy (15, 33, 73). The resulting negative health consequences that accompany prolonged muscle disuse atrophy, such as a reduction in functional capacity (15, 33), insulin sensitivity (62), and basal metabolic rate (42), have been well documented. As such, muscle disuse atrophy is of great clinical relevance, and therefore, it represents an important area for scientific investigation.

Although a single bout of prolonged disuse provides an acute metabolic and functional challenge to any individual, perhaps of more long-term relevance is the prevalence of (multiple) shorter periods of disuse that occur throughout the lifespan. For example, the average length of hospitalization for elderly patients admitted with acute illness is 5–6 days (29), and most periods of illness and injury that require home-based recovery/reduced physical activity generally last less than 1 wk. We (66, 68) and others (6, 25) have hypothesized that the accumulation of such short periods of disuse over the lifespan represents an important factor in the development of age-related sarcopenia. In support of this, we recently reported that even a period of muscle disuse lasting only 5 days already leads to substantial losses of skeletal muscle mass and functional strength in young (66) and older (17) subjects.

Mechanistically, any sizeable loss of skeletal muscle mass must be underpinned by a persistent imbalance between muscle protein synthesis and breakdown rates. A body of early muscle atrophy studies performed in animal models demonstrated comprehensively that a decline in muscle protein synthesis rates was a key factor responsible for disuse atrophy (e.g., see Refs. 36, 37, and 49). Critically, however, no data are currently available assessing in vivo muscle protein turnover rates during a period of short-term disuse (i.e., <10 days) in humans. Accordingly, the physiological mechanisms underlying skeletal muscle atrophy during short-term disuse in humans remain to be elucidated. Previous human studies, applying more prolonged periods of disuse, suggest that declines in the fasting muscle protein synthesis rates was a key factor responsible for disuse atrophy (e.g., see Refs. 36, 37, and 49). Critically, however, no data are currently available assessing in vivo muscle protein turnover rates during a period of short-term disuse (i.e., <10 days) in humans. Accordingly, the physiological mechanisms underlying skeletal muscle atrophy during short-term disuse in humans remain to be elucidated. Previous human studies, applying more prolonged periods of disuse, suggest that declines in the fasting muscle protein synthesis rates was a key factor responsible for disuse atrophy (e.g., see Refs. 36, 37, and 49).
following 5 days of leg immobilization in 12 healthy young men.

MATERIALS AND METHODS

Subjects. Twelve healthy young men (22 ± 1 yr) volunteered to participate in the present study. The subjects’ characteristics are presented in Table 1. All subjects were fully informed of the nature and possible risks of the experimental procedures before providing written informed consent. Subjects were screened to exclude any person with lower limb and/or back injuries sustained within 1 yr prior to the study, a (family) history of thrombosis/cardiovascular disease, use of anticoagulants, musculoskeletal/orthopaedic/hemostatic disorders, or participation in any regular resistance training program within 6 mo prior to the study. During screening, body composition (fat, fat-free mass, and bone mineral content) was determined by dual-energy X-ray absorptiometry scan (Discovery A, QDR Series; Hologic). Whole body and regional lean mass and percent body fat were determined using the software package Apex version 2.3 (Hologic). The study was approved by the Medical Ethics Committee of the Maastricht University Medical Centre (Maastricht, The Netherlands) in accordance with the guidelines set out in the Declaration of Helsinki.

Experimental design. Subjects participated in a single stable isotope tracer infusion experiment visit immediately following a 5-day period of one-legged knee immobilization by means of a full leg cast. During the experimental visit, continuous intravenous infusions of L-[ring-2H5]phenylalanine and L-[1-13C]leucine were combined with the ingestion of 25 g of intrinsically L-[1-13C]phenylalanine- and L-[1-13C]leucine-labeled dietary protein. Throughout the experimental visit, multiple blood samples were drawn and muscle samples obtained from both legs in a previously validated, comprehensive approach to determine muscle protein metabolism (7). This design allowed us to simultaneously assess postabsorptive and postprandial muscle protein synthesis rates and the metabolic fate of the ingested protein in the immobilized and nonimmobilized control leg.

Pretesting. Two days prior to the immobilization period, subjects participated in a single pretesting session to assess skeletal muscle mass of the limbs. Subjects arrived at the laboratory at 0800, and body weight was measured with a digital balance with an accuracy of 0.1 kg (SECA, Hamburg, Germany). Thereafter, a single-slice CT scan (Philips Brilliance 64; Philips Medical Systems, Best, The Netherlands) was performed to assess upper leg muscle cross-sectional area (CSA). The scanning characteristics were as follows: 120 kV, 300 mA, rotation time of 0.75 s, and a field of view of 500 mm. With subjects lying supine with their legs extended and feet secured, a 3-mm thick axial image was taken 15 cm proximal to the top of the patella. The precise scan position was marked with semipermanent ink for the duration of the experimental protocol to ensure accurate repeat measurements. Muscle area of the legs was selected between 0 and 100 Hounsfield units (31), after which the quadriceps muscle was selected by manual tracing using ImageJ software (version 1.45d; National Institutes of Health, Bethesda, MD) (30, 39, 61).

Table 1. Subject characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
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<tr>
<td>Age, yr</td>
<td>22 ± 1</td>
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<tr>
<td>Body mass, kg</td>
<td>82.5 ± 2.7</td>
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<tr>
<td>BMI, kg/m²</td>
<td>24.6 ± 0.7</td>
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<tr>
<td>Body fat, %/body mass</td>
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<tr>
<td>Lean body mass, kg</td>
<td>63.5 ± 1.6</td>
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<td>Average leg lean mass, kg</td>
<td>10.9 ± 0.4</td>
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<tr>
<td>Hb A1c, %</td>
<td>5.0 ± 0.1</td>
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Values represent means ± SE; n = 12. BMI, body mass index; Hb A1c, glycosylated hemoglobin.

Limb immobilization. Two days following pretesting, subjects attended the casting room at Maastricht University Medical Centre at 0800 to have a full leg cast fitted to induce one-legged knee immobilization. First, a high-moisture zinc paste bandage (Varicex T; Lohmann & Rauscher) was placed around the calf to prevent the cast dropping, and a foam ankle band was applied (Collar ‘n’ Cuff; Mölnlycke Health Care, Gothenburg, Sweden) to avoid pressure from the cast on the ankle joint. Thereafter, cotton padding was applied to the leg for skin protection (Cellona; Lohmann & Rauscher, Neuwied, Germany), and a fiberglass and polyurethane resin lightweight cast (Delta-Lite; BSN Medical) was fitted to the leg. The circular leg cast extended from 10 cm above the ankle to ~25 cm above the patella. The knee was casted at a 30° angle of flexion to prevent subjects performing any weight bearing on the casted limb. Subjects were provided with crutches for proper ambulation. Throughout the immobilization period, subjects were instructed to perform a series of daily simple ankle exercises (i.e., plantar and dorsal flexion and circular movements of the entire foot) to keep the calf muscle pump activated in the immobilized leg, thereby minimizing the risk of developing a deep vein thrombosis. Prior to the start of the stable isotope infusion visit, subjects visited the casting room to have the cast removed and had a second CT scan performed as described above. Following cast removal, subjects were transported exclusively by wheelchair to prevent any weight bearing on the immobilized leg.

Diet and physical activity. All subjects received the same standardized meal on the evening prior to the stable isotope infusion experimental visits [33 ± 2 g/kg body wt, providing 44 energy% (En%) carbohydrate, 22 En% protein, and 34 En% fat]. All volunteers were instructed to refrain from alcohol intake and keep their diet as constant as possible for the duration of the immobilization period.

Experimental visits. On the morning following the 5-day immobilization period (i.e., day 6), subjects arrived at the laboratory by taxi at 0800 for a stable isotope infusion experiment. Following cast removal and CT scanning (described above), a polytetrafluoroethylene catheter was inserted into an antecubital vein for stable isotope infusion. A second catheter was inserted into a heated dorsal hand vein of the contralateral arm, after which the hand was placed in a hot box (60°C) for arterialized blood sampling (1). After a basal blood sample was collected (t = −240 min), the plasma phenylalanine and leucine pools were primed with a single intravenous dose (2.0 μmol/kg L-[ring-2H5]phenylalanine, 4.0 μmol/kg L-[1-13C]leucine), after which continuous L-[ring-2H5]phenylalanine (0.06 μmol·kg⁻¹·min⁻¹) and L-[1-13C]leucine (0.10 μmol·kg⁻¹·min⁻¹) infusions were started. After the subjects rested in a semisupine position for 120 min, a second blood sample was drawn, and muscle biopsies were collected from the vastus lateralis muscle of both legs (t = −120 min). This signified the beginning of a 2-h period for the determination of postabsorptive muscle protein synthesis rates, during which blood samples were collected every 30 min (i.e., t = −90, −60, −30, and 0 min). At t = 0 min, muscle biopsies were again collected from the vastus lateralis muscle of both legs. Immediately after these biopsies, subjects ingested a single bolus of 25 g of intrinsically L-[1-13C]phenylalanine- and L-[1-13C]leucine-labeled whey protein in the immobilized and nonimmobilized control leg. We have shown previously (7) that the above-described use of doubly labeled milk protein allows the measurement of postprandial muscle protein synthesis rates without the disturbance of steady-state tracer conditions, with the concomitant determination of de novo muscle protein accretion from the ingested protein source (for full details of this approach, please refer to Ref. 7).

Following protein ingestion, subjects rested in a semisupine position for another 240 min, during which arterialized blood samples...
were collected at regular intervals (t = 30, 60, 90, 120, 180, and 240 min), after which final muscle biopsies were collected from both legs (t = 240 min), marking the end of the infusion period.

Blood samples were collected into EDTA-containing tubes and centrifuged at 3,500 g for 10 min at 4°C. Aliquots of plasma were frozen in liquid nitrogen and stored at −80°C. The muscle biopsy samples were all taken from separate incisions and in different directions ≥2 cm apart. Muscle biopsy samples were obtained from the middle region of the vastus lateralis, ~15 cm above the patella and ~3 cm below entry through the fascia, using the percutaneous needle biopsy technique (3). Muscle samples were dissected carefully and freed from any visible nonmuscle material and were immediately frozen in liquid nitrogen and stored at −80°C until further analysis.

Production of intrinsically labeled protein. Intrinsically, t-[1-13C]phenylalanine and t-[1-13C]leucine milk protein were obtained by a constant infusion of t-[1-13C]phenylalanine (455 μmol/min) and t-[1-13C]leucine (200 μmol/min) maintained for 96 h in a lactating dairy cow (9, 55, 65).

The milk was collected, processed, and fractionated into the whey protein concentrate, as described previously (55). The L-[1-13C]phenylalanine and L-[1-13C]leucine labeling of the myofibrillar proteins GC-C-IRMS analysis (Trace GC Ultra, IRMS model MAT 253; Thermo Scientific, Bremen, Germany). The derivatized amino acids were separated on a 30 m × 0.25 mm × 0.25 μm DB-5 column (temperature program: 120°C for 10 min, 3°C/min ramp to 200°C, and 30°C/min ramp to 300°C; hold for 5 min) prior to combustion. Standard regression curves were applied from a series of known standard enrichment values against the measured values to assess the linearity of the mass spectrometer and to account for any isotope fractionation that may have occurred during the analysis. In addition, Western blot homogenates (see below) were used to determine the muscle intracellular free amino acid pool tracer enrichments using previously described methods (7).

mRNA analyses. Total RNA was isolated from 10–20 mg of frozen muscle tissue using TRIzol Reagent (Life Technologies, Invitrogen) according to the manufacturer’s protocol. Total RNA quantification was carried out spectrophotometrically at 260 nm (NanoDrop ND-1000 Spectrophotometer; Thermo Fisher Scientific), and RNA purity was determined as the ratio of readings at 260/280 nm. Thereafter, first-strand cDNA was synthesized from 1 μg of RNA sample using the iScript cDNA synthesis kit (cat. 170-8891; Bio-Rad Laboratories). Taqman PCR was carried out using a 7300 Real Time PCR System (Applied Biosystems, Foster City, CA) with 2 μl of cDNA, 12.5 μl of Taqman master mix, 1.25 μl of Taqman probe, and 9.25 μl of H2O in a 25-μl final well volume. Each sample was run in duplicate together with a serial dilution standard curve. The housekeeping gene 18S was used as an internal control, as this gene was unaffected by immobilization (mean Ct values were unaffected over time; data not shown) and was used previously in similar studies (12, 43, 66). Taqman primer/probe sets were obtained from Applied Biosystems: t-type amino acid transporter 1 (LAT1), proton-assisted amino acid transporter 1 (PAT1), sodium-coupled neutral amino acid transporter 2 (SNAT2), CD98/SLC3A2 (CD98), muscle atrophy F-box (MAFBx), sodium-coupled neutral amino acid transporter 1 (LAT1), proton-assisted amino acid transporter 2 (SNAT2), CD98/SLC3A2 (CD98), muscle atrophy F-box (MAFBx), muscle RING finger-1 (Murf1), forkhead box O1 (FOXO1), IL-6, TNFα, and 18S. The thermal cycling conditions used were 2 min at 50°C and 10 min at 95°C, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Ct values of the target genes were normalized to Ct values of the internal control, and final results were calculated as relative expression against the standard curve. To avoid redundancy, mRNA data in the fasted state are presented only in the second fasting biopsy.

Western blotting. A portion of each muscle sample frozen for biochemical analyses was homogenized in seven volumes of Tris buffer (20 mM Tris-HCl, 5 mM EDTA, 10 mM Na-pyrophosphate, 100 mM NaF, 2 mM Na3VO4, and 1% Nonidet P-40, pH 7.4) supplemented with the following protease and phosphatase inhibitors: 10 μg/ml aprotonin, 10 μg/ml leupeptin, 3 mM benzamidine, and 1 mM PMSF. After homogenization, each muscle extract was centrifuged for 10 min at 10,000 g (4°C), and sample buffer was added to the supernatant to final concentrations of 60 mM Tris, 10% glycerol, 20 mg/ml SDS, 0.1 mM DTT, and 20 μg/ml bromophenol blue. The supernatant was then heated for 5 min at 100°C and immediately placed on ice. Immediately before analyses, the muscle extraction sample was warmed to 50°C and centrifuged for 1 min at 13,000 g (room temperature (RT)). Total amount of sample loaded on the gel was based on weight (1.0 mg/lane). With the exception of mammalian
target of rapamycin (mTOR), protein samples were run on a Criterion Precast TGX 4–20% gel (Bio-Rad order no. 567-1094) for 10 min at 50 V (constant voltage) and ±90 min at 150 V (constant voltage) and transferred onto a Trans-blot Turbo 0.2-μm nitrocellulose membrane (Bio-Rad order no. 170-4159) for 7 min at 2.5 A and 25 V. mTOR proteins were run and blotted under the same conditions but on a Criterion Precast XT 3–8% Tris-acetate gel (Bio-Rad order no. 345-0130). Specific proteins were detected by overnight incubation at 4°C on a shaker with specific antibodies in 50% PBS Odyssey blocking buffer (part no. 927-40000; LI-COR Biosciences, Lincoln, NE) after blocking for 60 min at RT in 50% PBS Odyssey blocking buffer. Polyclonal primary phosphospecific antibodies (α-tubulin (52 kDa, 1:10,000 dilution, rabbit monoclonal IgG, order no. 2125), anti-phospho-mTOR (Ser2448), anti-phospho-S6 protein kinase 1 (S6K1; Thr389), anti-phospho-S6 (Ser235/Ser236), anti-phospho-eukaryotic translation initiation factor 4E-binding protein-1 (4E-BP1; Thr37/46), anti-mTOR, anti-S6K1, anti-ribosomal protein S6 (RS6), and anti-4E-BP1) were purchased from Cell Signaling Technology (Beverly, MA). Following incubation, membranes were washed three times for 10 min in 0.1% PBS-Tween 20 and once for 10 min in PBS. Next, samples were incubated on a shaker (1 h at RT) with infrared secondary antibodies, donkey anti-rabbit IRDYE 800 (cat. no. 611-732-127, 1:10,000 dilution; Rockland), and donkey anti-mouse IRDYE 800CW (cat. no. 626-32212, 1:10,000 dilution; LI-COR Biosciences) dissolved in 50% PBS Odyssey blocking buffer. After a final wash step (3 × 10 min) in 0.1% Tween 20-PBS and once for 10 min in PBS, protein quantification was performed by scanning on an Odyssey Infrared Imaging System (LI-COR Biotechnology, Lincoln, NE), with local background subtraction and intensity of the bands expressed as arbitrary units. α-Tubulin was used to standardize for the amount of protein loaded. Phosphorylation status as a proxy of activation of the signaling proteins was expressed relative to the total amount of each protein.

Calculations. The fractional synthetic rates (FSRs) of the myofibrillar proteins were calculated using the standard precursor-product equation FSR (%/h) = ΔEp/Eprecursor × t × 100, where ΔEp is the change in phenylalanine or leucine labeling between two muscle biopsies, Eprecursor is the average labeling over time curve of the plasma precursor pool, and t indicates the tracer incorporation time (h) between two muscle biopsies.

Statistics. All data are expressed as means ± SE. A one-way ANOVA was used to assess changes over time in plasma glucose, insulin, and amino acid concentrations. A two-way repeated-measures ANOVA with time (pre and post) and leg (immobilized and nonimmobilized) as within-subjects factors was used to compare differences in quadriceps CSA. A two-way repeated-measures ANOVA with time (fasting and fed) and leg (immobilized and nonimmobilized) as within-subjects factors was used to compare differences in l-[1-13C]leucine and l-[ring-2H5]phenylalanine myofibrillar protein-bound enrichments and FSR and all mRNA and protein expression data. Myofibrillar protein bound l-[1-13C]phenylalanine enrichments and FSR were compared using paired t-tests. For all ANOVAs, when a significant time × leg interaction was detected, a Bonferroni post hoc test was applied to locate the individual differences. Statistical significance was set at P < 0.05. All calculations were performed by using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA).

RESULTS

Muscle mass. Muscle quadriceps CSA determined by CT scan of the midthigh is displayed in Fig. 1. Quadriceps CSA did not differ between legs at baseline and remained unchanged throughout the experiment in the nonimmobilized leg (from 8,039 ± 326 to 8,181 ± 313 mm², P = 0.24). However, 5 days of immobilization resulted in a 3.9 ± 0.6% decrease in quadriceps muscle CSA of the immobilized leg (from 8,208 ± 312 to 7,876 ± 285 mm², P < 0.0001).

Plasma analyses. Plasma glucose concentrations declined over time (P < 0.001) during the experimental visit (not shown). Plasma insulin concentrations (Fig. 2A) showed a rapid and brief increase following protein ingestion up to ~23 μU/l after 30 min before returning to baseline levels after 90 min (time effect, P < 0.001). Plasma phenylalanine (Fig. 2B), leucine (Fig. 2C), and tyrosine (Fig. 2D) concentrations increased (all P < 0.0001) following protein ingestion and remained above basal levels for 90–180 min. The time course of plasma l-[ring-2H5]phenylalanine (Fig. 2E), l-[1-13C]leucine (Fig. 2F), and l-[1-13C]phenylalanine (Fig. 2G) enrichments is also illustrated. During the postabsorptive period, plasma l-[ring-2H5]phenylalanine and l-[1-13C]leucine remained at steady state at ~7 and ~6 MPE, respectively. Following protein ingestion (at t = 0 min), plasma l-[ring-2H5]phenylalanine enrichments decreased for 90 min before returning to fasting, steady-state levels (P < 0.0001), whereas plasma l-[1-13C]leucine enrichments increased and remained at a steady state of ~8 MPE throughout the entire postprandial period (P < 0.0001). Following protein ingestion, plasma l-[1-13C]phenylalanine enrichments increased rapidly to ~14 MPE after 30 min and remained elevated for 180 min into the postprandial period (P < 0.0001). These higher plasma enrichments of l-[1-13C]phenylalanine compared with l-[1-13C]leucine following protein ingestion reflects the greater enrichment of the l-[1-13C]phenylalanine tracer in the intrinsically labeled protein. The greater rise in plasma leucine concentrations compared with phenylalanine is due to the higher leucine content (compared with phenylalanine) in whey protein.

Skeletal muscle tracer analyses. Based on the l-[ring-2H5]phenylalanine tracer and the plasma enrichment as the precursor pool, postabsorptive and postprandial myofibrillar FSRs were 41 ± 13 (0.015 ± 0.002 vs. 0.032 ± 0.005%/h, P < 0.01) and 53 ± 4% lower (0.020 ± 0.002 vs. 0.044 ± 0.003%/h, P < 0.01), respectively, in the immobilized compared with the control leg. Protein ingestion increased myofibrillar FSR in both legs (P < 0.05), and when Δchange was compared a trend was observed for a lower increase in the immobilized leg (P = 0.12).
Individual postabsorptive and postprandial myofibrillar FSRs based on the $\text{l-}[\text{ring-}^{2}\text{H}_{5}]$phenylalanine tracer and plasma precursor are illustrated in Fig. 3. Similar results were observed when the intracellular $\text{l-}[\text{ring-}^{2}\text{H}_{5}]$phenylalanine enrichments were used as the precursor pool, with postabsorptive and postprandial myofibrillar FSRs being 30 ± 19 (0.026 ± 0.004 vs. 0.051 ± 0.008%/h, $P < 0.01$) and 48 ± 4% lower (0.032 ± 0.003 vs. 0.062 ± 0.005%/h, $P < 0.01$), respectively, in the immobilized compared with the control leg. Postprandial FSR calculations using $\text{l-}[\text{ring-}^{2}\text{H}_{5}]$phenylalanine required the use of a modestly disturbed precursor pool (Fig. 2E). However, comparable postabsorptive and postprandial myofibrillar FSRs were observed when using the $\text{l-}[1-^{13}\text{C}]$leucine tracer, which, following a readjustment in enrichment levels during the transition from fasted to fed, presented a steady-state plasma precursor pool in both conditions (Fig. 2F). Specifically, postabsorptive (0.011 ± 0.012 vs. 0.036 ± 0.011%/h, $P < 0.01$) and postprandial (0.023 ± 0.003 vs. 0.055 ± 0.007%/h, $P < 0.01$) myofibrillar FSRs were lower in the immobilized compared with control leg, respectively. Moreover, similar results were observed when the intracellular $\text{l-}[1-^{13}\text{C}]$leucine enrichments were used as the precursor pool, with postabsorptive (0.018 ± 0.004 vs. 0.053 ± 0.015%/h, $P < 0.01$) and postprandial (0.044 ± 0.007 vs. 0.102 ± 0.014%/h, $P < 0.01$) myofibrillar FSRs being lower in the immobilized compared with control leg, respectively. Following protein ingestion, the increase in myofibrillar protein-bound $\text{l-}[1-^{13}\text{C}]$phenylalanine enrichments was 53 ± 18% lower in the immobilized compared with the control leg (0.007 ± 0.002 and 0.015 ± 0.002 MPE, respectively, $P < 0.05$; Fig. 4).

**mRNA and cell signaling analyses.** The skeletal muscle mRNA expression of genes implicated in the regulation of intracellular amino acid transport is presented in Fig. 5. Muscle LAT1 mRNA expression (Fig. 5A) increased with protein ingestion ($P < 0.05$) in both legs (by 87 and 57% for control and immobilized leg, respectively), with no effect of immobilization. PAT1 (Fig. 5B) and SNAT2 (Fig. 5C) were not affected by protein ingestion or immobilization. Muscle CD98 mRNA expression (Fig. 5D) increased with protein ingestion ($P < 0.05$) in both legs (by 20 and 26% for control and immobilized leg, respectively), with no effect of immobilization. Muscle mRNA expression of genes involved in muscle protein breakdown and inflammation are shown in Fig. 6. MAFbx (Fig. 6A), MuRF1 (Fig. 6B), and FOXO1 (Fig. 6C) mRNA expression were all greater in the immobilized compared with control leg (all $P < 0.05$) but were not affected by protein ingestion. Muscle TNFα mRNA expression was not affected by protein ingestion or immobilization, and muscle IL-6 mRNA expression increased with protein ingestion ($P < 0.05$) but was not changed with immobilization. Figure 7 depicts the muscle phosphorylation status (presented as a ratio of phosphorylated to total protein) of key proteins involved in the initiation of muscle protein synthesis. Protein ingestion increased the phosphorylation status of RS6 (Fig. 7C) in both legs (time effect, $P < 0.01$), and a similar trend ($P = 0.14$) was also observed for S6K1 (Fig. 7B). There was an interaction effect for mTOR (Fig. 7A) and 4E-BP1 (Fig. 7D) (both $P < 0.05$) such that the immobilized leg in the fed state showed lower phosphorylation status when compared with the corresponding control leg or fasted value.

**DISCUSSION**

The present study demonstrates that the loss of quadriceps muscle mass observed during merely 5 days of muscle disuse can be attributed to a decline in basal myofibrillar protein synthesis rates and a reduced capacity of skeletal muscle tissue to utilize dietary protein-derived amino acids for de novo myofibrillar protein synthesis in healthy males.

The present study demonstrates that the (~4%) loss of quadriceps cross-sectional area induced by 5 days of muscle...
anabolic properties of amino acids may develop with prolonged disuse (19, 34, 72). However, determining muscle protein synthesis rates in response to the ingestion of a bolus of dietary protein has its technical difficulties. The measurement of fractional muscle protein synthesis rate requires precursor pool enrichments to remain in a relative steady state between serial muscle biopsy collections. However, the ingestion of a meal-like bolus of dietary protein disrupts tracer steady state by diluting the precursor pool. Previous workers have attempted to obviate this problem by coingesting free labeled amino acids (8), ingesting small repeated boluses of protein (46), or simply making non-steady-state calculations (72). All of these approaches have their specific methodological limitations when postprandial muscle protein synthesis rates are assessed following ingestion of a single bolus of dietary protein (7). Recently, we produced milk proteins intrinsically labeled with high l-[1-13C]phenylalanine enrichment levels (>30 MPE) and lower levels of l-[1-13C]leucine (~8–10 MPE). The high enrichment l-[1-13C]phenylalanine levels allow us to directly assess the use of dietary protein-derived phenylalanine for de novo muscle protein synthesis, whereas the lower l-[1-13C]leucine enrichment allows us to match the plasma l-[1-13C]leucine precursor pool enrichment during a primed, constant, intravenous l-[1-13C]leucine infusion. Using this approach, we have previously measured postprandial muscle protein synthesis rates under both steady-state and non-steady-state precursor pool conditions as well as assessed the metabolic fate of the ingested protein-derived amino acids (7). Here, we show that protein ingestion increased muscle protein synthesis rates above basal, postabsorptive values in the control leg (0.032 ± 0.005 vs. 0.044 ± 0.003; Fig. 3). Despite a rise in circulating plasma insulin and leucine concentrations that is known to be sufficient to support maximal protein anabolism (40, 52, 75) (Fig. 2), postprandial myofibrillar protein synthesis rates were >50% lower in the previously immobilized leg when compared with the control leg (Fig. 3). The lower postprandial muscle protein synthetic rates in the immobilized leg were observed independent of which tracer was used to calculate FSR. It should be noted, however, that protein ingestion did still increase FSR in both legs, and the Δ increase in FSR only tended to be lower in the immobilized leg. This suggests that the absolute rate of FSR was more affected than the relative

Skeletal muscle is maintained by the successive daily stimulation of muscle protein synthesis rates following food intake (57). Previous studies employing more prolonged disuse protocols have suggested that a reduced responsiveness to the
FSR response to food ingestion. However, the postprandial incorporation of dietary protein-derived [1-13C]phenylalanine in myofibrillar protein was more than 50% lower in the immobilized vs. the control leg (Fig. 5), supporting the concept that the anabolic response to feeding per se is diminished. Consequently, a few days of disuse is followed by rapid onset of anabolic resistance of muscle tissue to dietary protein ingestion, resulting in reduced postprandial myofibrillar protein accretion. Therefore, it is of important clinical relevance to evaluate strategies that can overcome disuse-induced anabolic resistance and preserve muscle mass during short periods of muscle disuse. Effective strategies could include changing the amount (54, 76), type (5, 45, 53, 70), or timing (41) of dietary protein administration or applying mimetics for physical activity such as neuromuscular electrical stimulation (18, 69) to attenuate muscle disuse atrophy (71). The fact that disused muscle is still responsive to protein ingestion implies that the anabolic response to feeding per se is diminished. Impairments could conceivably reside systemically [e.g., protein digestion and/or absorption (28, 53) or the postprandial hormonal response and subsequent microvascular perfusion (56, 63)] or locally at the muscle tissue level [e.g., muscle amino acid uptake (23) and/or intramuscular signaling (14, 32)]. Previously, we provided evidence to suggest that the responsible mechanisms for disuse-induced anabolic resistance are intracellular (72). Intracellular muscle amino acid transport requires the presence of specific amino acid transporter proteins. Several of these amino acid transporters, including L-type amino acid transporter 1 (LAT1), sodium-coupled neutral amino acid transporter 2 (SNAT2), and CD98/SLC3A2 (CD98), reside on the cell membrane, and are thought to work in concert to regulate intracellular amino acid flux. Proton-assisted amino acid transporter (PAT1) is found in the lysosomal membrane and is thought to be instrumental in exporting amino acids from the lysosomal lumen into the cytosol. LAT1, SNAT2, CD98, and PAT1 mRNA and/or protein expression have all been shown to increase and coincide with a rise in muscle protein synthesis following essential amino acid ingestion and/or a single bout of resistance-type exercise (13, 22, 23). Interestingly, the increased expression of LAT1 and SNAT2 following essential amino acid ingestion was shown to be blunted following 7 days of bed rest (19). Taken together, these data have led to the hypothesis that the amino acid transporter expression response to increased amino acid availability may regulate postprandial muscle protein synthesis (16, 23). In the present study, we observed no differences in amino acid transporter expression levels between the immobilized and control leg (Fig. 5). Our data confirm the responsiveness of amino acid transporter expression following protein ingestion but do not support the hypothesis that changes in amino acid transporter expression may be responsible for the local muscle disuse-induced anabolic resistance. However, it should be acknowledged that we present only gene expression of these transporters, and this may not necessarily reflect their protein content and/or cellular location and involvement in amino acid transport.

Postprandial stimulation of muscle protein synthesis rates is initiated by a phosphorylation cascade where mTOR and its downstream targets P70S6 protein kinase (P70S6K/S6K1), ribosomal protein S6 (RS6), and 4E-BP1 are of central importance (21, 44). Consistent with previous studies (e.g., see Refs. 19 and 35), we report that the ingestion of protein led to a modest general increase in the phosphorylation of these anabolic signaling proteins (Fig. 7). Interestingly, immobilization significantly impaired postprandial activation of mTOR and its downstream target 4E-BP1 such that they actually decreased
following protein ingestion, whereas P70S6K and its subsequent activation of S6K1 did not seem to be affected. Both the lack of robust feeding-induced increases in phosphorylation of these proteins in the control leg as well as the decrease in phosphorylation in the immobilized leg may be explained by our study design, which allowed the determination of anabolic signaling at a single postprandial time point 4 h following protein ingestion. Because of its role in initiating the translation process, peak stimulation of this signaling cascade generally occurs 1–2 h following protein ingestion (13, 19, 35, 74) and begins to subside thereafter (13, 19, 35, 74). Therefore, it may be speculated that peak stimulation was missed and that we are observing the residual activation of these anabolic proteins in the control leg, and the reduced phosphorylation in the disused leg represents a reduced transduction of the anabolic signal through mTOR/4E-BP1, impairing the assembly of the active elF4F complex and/or via the suppression of translation initiation (44). Consistent with these findings, we have observed recently that lipid-induced anabolic resistance is also characterized by an impairment of postprandial 4E-BP1 activation (60). Collectively, our data point to an intracellular signaling defect that likely underpins anabolic resistance under diverse circumstances. Future work aiming to elucidate the intracellular impairments underlying disuse-induced anabolic resistance would likely require earlier and more frequent biopsy points to capture rapid, transient changes and determine a detailed time course of intracellular responses to protein ingestion following disuse. Moreover, employing dynamic measures of intracellular amino acid flux is required to discriminate between anabolic signaling defects per se and simply a reduced amino acid supply to the intracellular signaling pathways following disuse. A final, interesting consideration in light of our cell signaling data is the inclusion of younger subjects. Using a retrospective database analysis, it has been shown recently that aging is associated with a hyperphosphorylation of mTOR in the fasted state (51). Moreover, it has also been reported that mTOR/P70S6K responsiveness to protein ingestion may be enhanced in older muscle (26) despite a reduced muscle protein synthetic response. These findings suggest a reduced efficiency of older muscle in terms of signal transduction or at least an altered dynamic relationship between cell signaling and muscle protein synthesis. In the same way, it could also be speculated that disuse may alter the efficiency and/or kinetics or feeding-induced mTOR activation, underlining the importance of conducting future time course-based studies. Consequently, understanding the relationship between the relevant signaling pathways and muscle protein synthesis in disused young and older muscle remains an important future research direction.

Fig. 6. Skeletal muscle mRNA expression of muscle atrophy F-box (MAFBx; A), muscle RING finger-1 (MuRF1; B), forkhead box O1 (FOXO1; C), TNFα (D), and IL-6 (E) in the fasted and fed (4 h following ingestion of 25 g of protein) states following 5 days of 1-legged knee immobilization in the IMMOB and CON legs of healthy young men (n = 12). Data were analyzed with 2-way repeated-measures ANOVAs. A: significant main effect of immobilization (P < 0.05). B and C: significant main effect of immobilization (P < 0.01). D: no significant effects. E: significant main effect of protein ingestion (P < 0.05). †P < 0.05 and ††P < 0.01 compared with corresponding CON values; *P < 0.05 compared with corresponding fasted value.
Aside from changes in muscle protein synthesis, we assessed the gene expression of two primary ubiquitin ligases, MAFbx and MuRF1, and their key transcription factor FOXO1 (4, 38) to obtain insight into possible changes in muscle protein breakdown. In keeping with our previous work (17, 66), the expression of these genes was markedly elevated in the immobilized compared with the control leg (Fig. 6). These data are consistent with previous assertions that a rapid and transient rise in muscle protein breakdown may contribute to muscle disuse atrophy (50, 64, 67, 73), although it should be noted that these static molecular data cannot be considered conclusive evidence for alterations in muscle protein breakdown. Indeed, recent reports have suggested that MuRF1 may also have a pivotal role in inhibiting muscle protein synthetic pathways (2) and could have contributed to the observed anabolic resistance. Although it has been suggested that a rapid rise in muscle protein breakdown may contribute to muscle disuse atrophy (50, 64, 67, 73), although it should be noted that these static molecular data cannot be considered conclusive evidence for alterations in muscle protein breakdown. Indeed, recent reports have suggested that MuRF1 may also have a pivotal role in inhibiting muscle protein synthetic pathways (2) and thus could have contributed to the observed anabolic resistance. Although it has been suggested that a rapid rise in muscle protein breakdown at the onset of disuse may be provoked by a proinflammatory state (24, 48), we failed to detect any evidence of increased inflammation in the immobilized muscle tissue as indicated by IL-6 and TNFα gene expression (Fig. 6). Irrespective of leg, protein ingestion resulted in a robust increase in IL-6 gene expression that likely reflects the shift in energy and substrate metabolism in the transition to the postprandial state, processes within which IL-6 plays a metabolic role (27, 59).

In conclusion, the present study demonstrates that a decline in postabsorptive myofibrillar protein synthesis rates and an impaired capacity to utilize dietary protein-derived amino acids for de novo myofibrillar protein synthesis are key factors underlying short-term skeletal muscle disuse atrophy. Given the proposed role of muscle disuse in the development of age-related sarcopenia, it is critical that strategies aimed at stimulating muscle protein synthesis rates and/or overcoming anabolic resistance should be developed and applied as early as possible in an effort to preserve skeletal muscle mass and function.

ACKNOWLEDGMENTS

We acknowledge Dr. Henrike Hamer for efforts in the initial setup of this project, Antoine Zorenc and Joy Goessens for their technical assistance, the enthusiastic support of the staff at the Maastricht University Hospital casting room, and the volunteers for their participation. This study was registered at the Netherlands Trial Register: NTR4060.

GRANTS

This project was funded by TF Food and Nutrition, a public/private partnership on precompetitive research in food and nutrition. The public partners are responsible for the study design, data collection and analysis, decision to publish, and preparation of the manuscript. The private partners have contributed to the project through regular discussion.

DISCLOSURES

None of the authors have any conflicts of interest or financial disclosures to declare.

AUTHOR CONTRIBUTIONS


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