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

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Abstract

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 five days of muscle disuse on post-absorptive and post-prandial myofibrillar protein synthesis rates in humans. Twelve healthy, young (22±1 y) men underwent a five 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 labelled dietary protein to assess myofibrillar muscle protein fractional synthetic rates (FSRs) in the immobilized and non-immobilized control leg. Immobilization led to a 3.9±0.6 % decrease in quadriceps muscle CSA of the immobilized leg. Based on the L-[^ring-2H5]phenylalanine tracer, immobilization reduced post-absorptive myofibrillar protein synthesis rates by 41±13% (0.015±0.002 vs 0.032±0.005 %h⁻¹; P<0.01) and post-prandial myofibrillar protein synthesis rates by 53±4% (0.020±0.002 vs 0.044±0.003 %h⁻¹; 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% lower in the immobilized compared with the control leg (0.007±0.002 and 0.015±0.002 MPE, respectively; P<0.05). We conclude that five days of muscle disuse substantially lowers post-absorptive myofibrillar protein synthesis rates and induces anabolic resistance to protein ingestion.
Introduction

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 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 one week. 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, we recently reported that even a period of muscle disuse lasting only five 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 comprehensively demonstrated that a decline in muscle protein synthesis rates was a key factor responsible for disuse atrophy (e.g. 36, 37, 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, suggests that declines in the fasting muscle protein synthesis rates (28, 33, 34) and/or a reduced responsiveness to the anabolic properties of amino acids/food intake (19, 34, 72) are important factors responsible for disuse atrophy. In the present study, we applied specifically produced intrinsically L-[1-\(^{13}\)C]phenylalanine and L-[1-\(^{13}\)C]leucine labelled dietary protein combined with continuous intravenous L-[ring-\(^{2}\)H\(_5\)]phenylalanine and L-[1-\(^{13}\)C]leucine infusions (7) to assess both post-absorptive and post-prandial muscle protein synthesis rates as well as the metabolic fate of ingested protein following 5 days of leg immobilization in 12 healthy young men.
Materials and Methods

Subjects

Twelve healthy, young (22±1 y) men volunteered to participate in the present study. 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 a year prior to the study, a (family) history of thrombosis/cardiovascular disease, use of anti-coagulants, musculoskeletal/orthopaedic/haemostatic disorders, or participation in any regular resistance training program within 6 months prior to the study. During screening, body composition (fat, fat-free mass and bone mineral content) were determined by dual-energy x-ray absorptiometry (DXA) scan (Hologic Inc., Discovery A, QDR series, Bradford, USA). Whole-body and regional lean mass and percent body fat were determined using the software package Apex version 2.3 (Hologic, Bedford, USA). 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 experimental visit immediately following a five 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 intrinsically L-[1-13C]phenylalanine and L-[1-13C]leucine labelled dietary protein. Throughout the experimental visit, multiple blood samples were drawn and muscle samples were obtained from both legs in a previously validated, comprehensive approach to determine muscle protein metabolism (7).
This design allowed us to simultaneously assess post-absorptive and post-prandial muscle protein synthesis rates, and the metabolic fate of the ingested protein, in the immobilized and non-immobilized control leg.

Pre-testing

Two days prior to the immobilization period, subjects participated in a single pre-testing session to assess skeletal muscle mass of the legs. Subjects arrived at the laboratory at 08.00 h and body weight was measured with a digital balance with an accuracy of 0.1 kg (SECA GmbH, 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 semi-permanent 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 Institute of Health, Maryland, USA) (30, 39, 61). Next, the leg to be immobilized was selected at random (counterbalanced for left and right legs) and subjects were instructed on, and familiarized with, the use of crutches (casting described below). On the morning of the experimental test day (i.e. the end of the immobilization period) the cast removal was immediately followed by a second CT scan.

Limb immobilization
Two days following pre-testing, subjects attended the Casting Room at Maastricht University Medical Centre at 08.00 h to have a full leg cast fitted to induce one-legged knee immobilization. First, a high moisture zinc paste bandage (Varicex®T, Lohmann & Rauscher, Germany) 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, Germany) was fitted to the leg. The circular leg cast extended from 10 cm above the ankle to approximately 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 exclusively transported by wheelchair to prevent any weight bearing on the immobilized leg.

**Diet and physical activity**

All subjects received the same standardized meal the evening prior to the stable isotope infusion experimental visits (33±2 kJ kg⁻¹ body weight, providing 44 energy% (En%) carbohydrate, 22 En% protein, and 34 En% fat). All volunteers were instructed to refrain from alcohol intake and to 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 08:00 h 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$^{-1}$ L-$[\text{ring}-^2\text{H}_3]$phenylalanine; 4.0 µmol·kg$^{-1}$ L-$[1^{-13}\text{C}]$leucine), after which continuous L-$[\text{ring}-^2\text{H}_3]$phenylalanine (0.06 µmol·kg$^{-1}$·min$^{-1}$) and L-$[1^{-13}\text{C}]$leucine (0.10 µmol·kg$^{-1}$·min$^{-1}$) infusions were started. After the subjects rested in a semi-supine 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 post-absorptive 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 intrinsically L-$[1^{-13}\text{C}]$phenylalanine and L-$[1^{-13}\text{C}]$leucine-labeled whey protein dissolved in 350 mL vanilla flavored water signifying the beginning of a 4 h period for the determination of post-prandial muscle protein synthesis rates. We have previously shown (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 the approach please refer to; 7).
Following protein ingestion, subjects rested in a semi-supine 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 3500g 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, at least 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 non-muscle material and were immediately frozen in liquid nitrogen and stored at -80°C until further analysis.

Production of intrinsically labelled protein

Intrinsically L-[1-13C]phenylalanine and L-[1-13C]leucine milk protein was obtained by a constant infusion of L-[1-13C]phenylalanine (455 µmol/min) and L-[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 previously described (55). The L-[1-13C]phenylalanine and L-[1-13C]leucine enrichments in whey protein were measured by gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS; MAT 252, Finnigan, Breman, Germany) and averaged 38.7 MPE and 9.3 MPE, respectively. The proteins met all chemical and bacteriologic specifications for human consumption.

Plasma analyses
Plasma glucose and insulin concentrations were analyzed using commercially available kits (Glucose HK CP, ABX Diagnostics, ref. A11A01667, Montpellier, France and Millipore, ref. HI-14K, Billerica, USA, respectively). Plasma amino acid concentrations and enrichments were determined by GCMS analysis (Agilent 7890A GC/5975C; MSD, Little Falls, DE, USA). Specifically, internal standards of [U-13C6]leucine and [U-13C915N]phenylalanine, and [U-13C915N]tyrosine were added to the samples. The plasma was deproteinised on ice with 10 mg of dry 5-sulfosalicylic acid. Free amino acids were purified using cation exchange chromatography (AG 50W-X8 resin; Bio-Rad Laboratories, Hercules, CA, USA). The free amino acids were converted to their t-butyldimethylsilyl (TBDMS) derivative before analysis by GCMS. The amino acid concentrations were determined using electron impact ionization by monitoring ions at mass/charge ($m/z$) 302 and 308 for unlabeled and [U-13C6]leucine respectively, 336 and 346 for unlabeled and [U-13C915N]phenylalanine respectively, and 466 and 476 for unlabeled and [U-13C915N]tyrosine respectively. The plasma free L-[ring-$^2$H5]phenylalanine enrichments were measured from the $m/z$ ratios at 336 ($m+0$) and 341 ($m+5$), with $m+0$ representing the lowest molecular weight of the ion. For L-[1-13C]phenylalanine, $m/z$ 336 ($m+0$) and 337 ($m+1$) were monitored. For L-[1-13C]leucine, $m/z$ 302 ($m+0$) and 303 ($m+1$) were monitored. Standard regression curves were applied in all isotopic enrichment analyses to assess linearity of the mass spectrometer and to control for the stable isotope effects that occur during analysis. Phenylalanine and leucine enrichments were corrected for the presence of both the $^{13}$C and $^2$H isotopes.

Muscle tracer analyses

Myofibrillar protein enriched fractions were extracted from 50 mg of wet muscle tissue by hand-homogenizing on ice using a Teflon pestle in a standard extraction buffer (7.5 $\mu$l/mg$^{-1}$) (47). The samples were spun at 1500g for 10 min at 4°C. The resultant supernatants that
contained the sarcoplasmic proteins were removed. The remaining myofibrillar and collagen pellet were washed with 500 µl of extraction buffer and spun at 700 ×g for 10 min at 4˚C. The myofibrillar proteins were solubilized by adding 1.5 mL of 0.3 м NaOH and heating at 50˚C for 30 min with vortex mixing every 10 min. Samples were centrifuged at 10000g for 5 min at 4˚C, the supernatant containing the myofibrillar-enriched fraction was collected and the collagen pellet was discarded. Myofibrillar proteins were precipitated by the addition of 1 mL of 1 м PCA and spinning at 700g for 10 min at 4˚C. The myofibrillar enriched proteins were washed twice with 70% ethanol. Amino acids were liberated by the addition of 2 mL of 6 м HCL and heating at 110˚C for 24 h. The hydrolysed myofibrillar protein fraction was dried under a nitrogen stream while being heated to 120˚C and, subsequently, dissolved in a 25% acetic acid solution and passed over a Dowex exchange resin (AG 50W-X8, 100 – 200 mesh hydrogen form; Biorad, Hercules, CA, USA). The free amino acids were eluted with 2 м NH₄OH, dried, and the purified amino acids were derivatised into MTBSTFA-phenylethylamines (11, 58) to measure the L-[ring-²H₅]phenylalanine labelling using GCMS analysis as described previously (10). Separate aliquots of the purified amino acids were converted to their N(O,S)-ethoxycarbonyl ethyl esters derivatives to determine the L-[1-¹³C]phenylalanine and L-[1-¹³C]leucine labelling of the myofibrillar proteins by gas chromatography combustion-isotope ratio mass spectrometry analysis (GC-C-IRMS; Trace GC Ultra, IRMS model MAT 253, Thermo Scientific, Breman, Germany). The derivatised amino acids were separated on a 30m×0.25mm×0.25µm DB-5 column (temperature program: 120˚C for 10 min; 3˚C min⁻¹ ramp to 200˚C; 30˚C min⁻¹ 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 methods described previously (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, USA), and RNA purity was determined as the ratio of readings at 260/280 nm. Thereafter, first strand cDNA was synthesized from 1 µg RNA sample using iScript™ cDNA synthesis kit (BioRad; cat. 170-8891). Taqman PCR was carried out using a 7300 Real Time PCR System (AppliedBiosystems, USA), with 2 µL of cDNA, 12.5 µl Taqman™ master mix, 1.25 µl Taqman™ probe and 9.25 µl 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 has been used previously in similar studies (12, 43, 66). Taqman primer/probe sets were obtained from Applied Biosystems (Foster City, USA): LAT1, PAT1, SNAT2, CD98, MAFBx, MuRF1, FOXO1, IL-6, TNFα and 18S. The thermal cycling conditions used were: 2 min at 50°C, 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 only presented in the second fasting biopsy.
Western blotting

A portion of each muscle sample frozen for biochemical analyses was homogenized in 7 volumes Tris buffer (20 mM Tris-HCl, 5 mM EDTA, 10 mM Na-pyrophosphate, 100 mM NaF, 2 mM Na3VO4, 1% Nonident P-40; pH 7.4) supplemented with the following protease and phosphatase inhibitors: Aprotinin 10 µg/mL, Leupeptin 10 µg/mL, Benzamidin 3 mM and PMSF 1 mM. After homogenization, each muscle extract was centrifuged for 10 min at 10,000g (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, 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 13,000g (RT). Total amount of sample loaded on the gel was based on weight (1.0 mg per lane). With the exception of mTOR, protein samples were run on a Criterion Precast TGX 4-20% gel (Biorad 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 (Biorad Order No. 170-4159) in 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 (Biorad order No. 345-0130). Specific proteins were detected by overnight incubation at 4°C on a shaker with specific antibodies in 50% in PBS Odyssey blocking buffer (Li-Cor Biosciences Part No. 927-40000) after blocking for 60 min at RT in 50% in PBS Odyssey blocking buffer. Polyclonal primary phospho-specific antibodies [α-tubulin (52 kD; dilution 1:10 000, rabbit monoclonal IgG; Cell Signaling Tecnologies, Beverly, MA, USA; Order No. 2125), anti-phospho-mTOR (Ser2448), anti-phospho-S6K1 (Thr389), anti-phospho-S6 (Ser235/Ser236), anti-phospho-4E-BP1 (Thr37/46), anti-mTOR, anti-S6K1, anti-RS6 and anti-4E-BP1 were purchased from Cell Signaling Technologies. Following incubation, membranes were washed 3 times 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 (Rockland, Cat. No. 611-732-127, dilution 1:10000) and donkey anti-mouse IRDYE 800CW (Li-Cor, Cat. No. 626-32212, dilution 1:10000) dissolved in 50% PBS Odyssey blocking buffer. After a final wash step (3 x 10 min) in 0.1% Tween20-PBS and once 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:

\[
\text{FSR} \quad \text{(%h}^{-1}\text{)} = \frac{\Delta E_p}{E_{\text{precursor}} \times t} \times 100
\]

where \(\Delta E_p\) is the change in phenylalanine or leucine labelling between two muscle biopsies, \(E_{\text{precursor}}\) is the average labelling over time curve (AUC) of the plasma precursor pool; and \(t\) indicates the tracer incorporation time (hours) between two muscle biopsies.

**Statistics**

All data are expressed as means±SEM. 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 non-immobilized) 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 non-immobilized) as within-subjects factors was used to compare differences in L-[1-^{13}\text{C}]leucine and L-[ring-
$^{2}$H$_5$]phenylalanine myofibrillar protein bound enrichments and FSR and all mRNA and protein expression data. Myofibrillar protein bound L-$[1^{-13}$C]phenylalanine enrichments and FSR were compared using paired $t$-tests. For all ANOVA analyses, when a significant time*leg interaction was detected, a Bonferoni 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, USA).
Results

Muscle mass

Muscle quadriceps cross sectional area (CSA) determined by CT-scan of the mid-thigh is displayed in Figure 1. Quadriceps CSA did not differ between legs at baseline and remained unchanged throughout the experiment in the non-immobilized leg (from 8039±326 to 8181±313 mm²; \( P=0.24 \)). However, five days of immobilization resulted in a 3.9±0.6% decrease in quadriceps muscle CSA of the immobilized leg (from 8208±312 to 7876±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 (Figure 2A) showed a rapid and brief increase following protein ingestion up to \( \sim 23 \) mU L\(^{-1} \) after 30 min, before returning to baseline levels after 90 min (time effect; \( P<0.001 \)). Plasma phenylalanine (Figure 2B), leucine (Figure 2C) and tyrosine (Figure 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-\( ^2 \)H\(_5 \)]phenylalanine (E), L-[1-\( ^{13} \)C]leucine (F) and L-[1-\( ^{13} \)C]phenylalanine (G) enrichments are also illustrated in Figure 2. During the post-absorptive period, plasma L-[ring-\( ^2 \)H\(_5 \)]phenylalanine and L-[1-\( ^{13} \)C]leucine remained in steady-state at \( \sim 7 \) and \( \sim 6 \) MPE, respectively. Following protein ingestion (at \( t=0 \) min), plasma L-[ring-\( ^2 \)H\(_3 \)]phenylalanine enrichments decreased for 90 min before returning to fasting, steady-state levels (\( P<0.0001 \)), while plasma L-[1-\( ^{13} \)C]leucine enrichments increased and remained at a steady state of \( \sim 8 \) MPE throughout the entire post-prandial period (\( P<0.0001 \)). Following protein ingestion, plasma L-[1-\( ^{13} \)C]phenylalanine enrichments increased rapidly to \( \sim 14 \) MPE after 30 min and...
remained elevated for 180 min into the post-prandial period \((P<0.0001)\). These higher plasma enrichments of L-[1-\(^{13}\)C]phenylalanine compared with L-[1-\(^{13}\)C]leucine following protein ingestion reflects the greater enrichment of the L-[1-\(^{13}\)C]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-[\(\text{ring}^{-2}\)H\(_5\)]phenylalanine tracer and the plasma enrichment as the precursor pool, post-absorptive and post-prandial myofibrillar FSRs were 41±13\% (0.015±0.002 vs 0.032±0.005 \%\,h\(^{-1}\); \(P<0.01\)) and 53±4\% (0.020±0.002 vs 0.044±0.003 \%\,h\(^{-1}\); \(P<0.01\)) lower, respectively, in the immobilized compared with the control leg. Protein ingestion increased myofibrillar FSR in both legs \((P<0.05)\), and when comparing delta change a trend was observed for a lower increase in the immobilized leg \((P=0.12)\). Individual post-absorptive and post-prandial myofibrillar FSRs based on the L-[\(\text{ring}^{-2}\)H\(_5\)]phenylalanine tracer and plasma precursor are illustrated in Figure 3. Similar results were observed when the intracellular L-[\(\text{ring}^{-2}\)H\(_5\)]phenylalanine enrichments were used as the precursor pool, with post-absorptive and post-prandial myofibrillar FSRs being 30±19 \% (0.026±0.004 vs 0.051±0.008 \%\,h\(^{-1}\); \(P<0.01\)) and 48±4\% (0.032±0.003 vs 0.062±0.005 \%\,h\(^{-1}\); \(P<0.01\)) lower, respectively, in the immobilized compared with the control leg. Post-prandial FSR calculations using L-[\(\text{ring}^{-2}\)H\(_5\)]phenylalanine required the use of a modestly disturbed precursor pool (Figure 2E). However, comparable post-absorptive and post-prandial myofibrillar FSRs were observed when using the L-[1-\(^{13}\)C]leucine tracer which, following a readjustment in enrichments levels during the transition from fasted to fed, presented a steady state plasma precursor pool in both conditions (Figure 2F). Specifically, post-absorptive (0.011±0.012 vs 0.036±0.011 \%\,h\(^{-1}\);
and post-prandial (0.023±0.003 vs 0.055±0.007 %·h⁻¹; \(P<0.01\)) myofibrillar FSRs were lower in the immobilized compared to control leg, respectively. Moreover, similar results were observed when the intracellular L-[1-\(^{13}\)C] leucine enrichments were used as the precursor pool, with post-absorptive (0.018±0.004 vs 0.053±0.015 %·h⁻¹; \(P<0.01\)) and post-prandial (0.044±0.007 vs 0.102±0.014 %·h⁻¹; \(P<0.01\)) myofibrillar FSRs being lower in the immobilized compared with control leg, respectively. Following protein ingestion, the increase in myofibrillar protein bound L-[1-\(^{13}\)C]phenylalanine enrichments were 53±18% lower in the immobilized compared with the control leg (0.007±0.002 and 0.015±0.002 MPE, respectively; \(P<0.05\); Figure 4).

mRNA and cell signaling analyses

The skeletal muscle mRNA expression of genes implicated in the regulation of intracellular amino acid transport are presented in Figure 5. Muscle LAT1 mRNA expression (A) 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 (B) and SNAT2 (C) were not affected by protein ingestion or immobilization. Muscle CD98 mRNA expression (D) 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 Figure 6. Muscle MAFBx (A), MuRF1 (B) and FOXO1 (C) 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 (C) in both legs (time effect; \( P<0.01 \)) and a similar trend (\( P=0.14 \)) was also observed for S6K1 (B). There was an interaction effect for mTOR (A) and 4-EBP1 (D) (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 five 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 disuse (Figure 1) is accompanied by an approximate halving of post-absorptive myofibrillar protein synthesis rates when compared with the non-immobilized, control leg (Figure 3). This striking decrease was comparable irrespective of whether the incorporation of L-[ring-2H5] phenylalanine or L-[1-13C]leucine was used to calculate myofibrillar protein synthesis rates. Our data demonstrate that the decline in basal muscle protein synthetic rate that occurs during more prolonged immobilization and bed-rest studies (28, 33, 34) occurs much more rapidly than previously thought. Extending on this, we report that the reduced capacity to synthesize muscle proteins in the post-absorptive state is pronounced in the contractile myofibrillar proteins. Therefore, reduced basal myofibrillar protein synthesis rates can, at least in part, explain the considerable loss of muscle mass and function that rapidly occurs during only a few days of muscle disuse. As such, increasing basal myofibrillar protein synthesis rates should form a key therapeutic target for effective interventional strategies aiming to preserve muscle mass.

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 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 disturbs tracer steady state by diluting the precursor pool. Previous workers have attempted to obviate this problem by co-ingesting free labelled amino acids (8), ingesting small repeated boluses of protein (46), or simply by making non-steady state calculations (72). All these approaches have their specific methodological limitations when assessing post-prandial muscle protein synthesis rates following ingestion of a single bolus of dietary protein (7). Recently, we produced milk proteins intrinsically labelled with high L-[1-\(^{13}\)C]phenylalanine enrichment levels (>30 MPE) and lower levels of L-[1-\(^{13}\)C]leucine (~8-10 MPE). The high enrichment L-[1-\(^{13}\)C]phenylalanine levels allow us to directly assess the use of dietary protein derived phenylalanine for \textit{de novo} muscle protein synthesis, whereas the lower L-[1-\(^{13}\)C]leucine enrichment allows us to match the plasma L-[1-\(^{13}\)C]leucine precursor pool enrichment during a primed constant intravenous L-[1-\(^{13}\)C]leucine infusion. Using this approach, we have previously measured post-prandial muscle protein synthesis rates under both steady and non-steady-state precursor pool conditions as well as assessing the metabolic fate of the ingested protein derived amino acids (7). Here, we show that protein ingestion increased muscle protein synthesis rates above basal, post-absorptive values in the control leg (0.032±0.005 vs 0.044±0.003; \textbf{Figure 3}). Despite a rise in circulating plasma insulin and leucine concentrations known to be sufficient to support maximal protein anabolism (\textbf{Figure 2}) (40, 52, 75), post-prandial myofibrillar protein synthesis rates were >50% lower in the previously immobilized leg when compared with the control leg (Figure 3). The lower post-prandial 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 delta 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 FSR response to food ingestion. However, the post-prandial incorporation of dietary protein derived [1-13C]Phenylalanine in myofibrillar protein was more than 50% lower in the immobilized versus control leg (Figure 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 post-prandial myofibrillar protein accretion. It is therefore 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 interventional strategies aiming to preserve muscle mass during a period of disuse should minimize time spent in the post-absorptive state.

Although anabolic resistance has now been reported in a variety of situations, the underlying mechanism(s) are poorly understood. Impairments could conceivably reside systemically (e.g. protein digestion and/or absorption (28, 53), or the post-prandial 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)). We have previously 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 post-prandial 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 (Figure 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 only present gene expression of these transporters, and this may not necessarily reflect their protein content and/or cellular location and involvement in amino acid transport.

Post-prandial stimulation of muscle protein synthesis rates is initiated by a phosphorylation cascade where mammalian target of rapamycin (mTOR) and its downstream targets P70S6 protein kinase (P70S6K/S6K1), ribosomal protein S6 (RS6) and eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1) are of central importance (21, 44). Consistent with previous studies (e.g. 19, 35) we report that the ingestion of protein led to a modest general increase in the phosphorylation of these anabolic signaling proteins (Figure 7). Interestingly, immobilization significantly impaired post-prandial 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 post-
prandial time-point, 4 h following protein ingestion. Due to its role in initiating the translation
process, peak stimulation of this signaling cascade generally occurs 1-2 h following protein
ingestion (13, 20, 35, 74) and begins to subside thereafter (13, 20, 35, 74). It may be
speculated, therefore, that peak stimulation was missed and 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 eIF4F complex and/or via the suppression of translation
initiation (44). Consistent with these findings, we have recently observed that lipid induced
anabolic resistance is also characterized by an impairment of post-prandial 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 are 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 recently been shown 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 also 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.

Aside from changes in muscle protein synthesis, we assessed the gene expression of two primary ubiquitin ligases, Muscle Atrophy F-Box/atrogin-1 (MAFbx) and Muscle-Specific RING-finger protein 1 (MuRF1), and their key transcription factor, fork head box protein 01 (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 were markedly elevated in the immobilized compared with the control leg (Figure 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), though it should be noted that these static molecular data cannot be considered as 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 pro-inflammatory 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 (Figure 6). Irrespective of leg, protein ingestion resulted in a robust increase in IL-6 gene expression which likely reflects the shift in energy and substrate metabolism in the transition to the post-prandial state, processes within which IL-6 plays a metabolic role (27, 59).
In conclusion, the present study demonstrates that a decline in post-absorptive 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.
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Author Contributions

The authors’ contributions were as follows: BTW, MLD, JWvD and LJCvL designed the research; BTW, MLD, TS and JWvD conducted the research; BTW carried out the statistical analyses; BTW, MLD and LJCvL wrote the manuscript.

BTW and MLD contributed equally to this work and should be considered as joint first authors.

All authors contributed to and approved the final version of the manuscript.

Competing Interests

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

This study was registered at the Netherlands Trial Register: NTR4060
References


**FIGURE LEGENDS**

**Figure 1** Mean (±SEM) quadriceps cross sectional area (CSA) before (PRE) and after (POST) 5 days of one-legged knee immobilization in the immobilized (IMMOB) and non-immobilized control (CON) legs of healthy, young men (n=12). Data were analyzed with a two-way repeated measures ANOVA (time x leg) with Bonferroni post-hoc tests to locate differences: Significant time (P<0.05) and interaction (P<0.01) effects, * = P<0.05 compared with corresponding PRE value.

**Figure 2** Mean (±SEM) plasma insulin (A), phenylalanine (B), leucine (C) and tyrosine (D) concentrations, and mean (±SEM) plasma L-[ring-2H5]phenylalanine (E), L-[1-13C]leucine (F) and L-[1-13C]phenylalanine (G) enrichments across time (x axis; minutes) before and following ingestion of 25 g protein (at t=0 min) after 5 days of one-legged knee immobilization in healthy, young men (n=12). Data were analyzed with a one-way repeated measures ANOVA with Bonferroni post hoc tests applied to locate differences: A, B, C, D, E, F and G all showed significant time effects (P<0.001) and * indicates values significantly different compared to ‘0’.

**Figure 3** Individual subjects’ post-absorptive (A) and post-prandial (B) fractional myofibrillar protein synthesis rates (FSR) calculated from L-[ring-2H5]phenylalanine following 5 days of one-legged knee immobilization in the immobilized (IMMOB) and non-immobilized control (CON) legs of healthy, young men (n=12). Data were analyzed with a two-way repeated measures ANOVA and significant main effects of protein ingestion (P<0.05) and immobilization (P<0.001) were detected (for interaction P=0.16).
Figure 4 Delta myofibrillar protein enrichment of L-[1-13C]phenylalanine 4 h after the ingestion of 25 g intrinsically L-[1-13C]phenylalanine labelled protein following 5 days of one-legged knee immobilization in the immobilized (IMMOB) and non-immobilized control (CON) legs of healthy, young men (n=12). Data were analyzed with a paired t-test. * = P<0.05 compared with CON.

Figure 5 Skeletal muscle mRNA expression of LAT1 (A), PAT1 (B), SNAT2 (C) and CD98 (D) in the fasted and fed (4 h following ingestion of 25 g protein) state following 5 days of one-legged knee immobilization in the immobilized (IMMOB) and non-immobilized control (CON) legs of healthy, young men (n=12). Data were analyzed with two-way repeated measures ANOVAs. A: Significant main effect of protein ingestion (P<0.05). B: No significant effects. C: No significant effects. D: Significant main effect of protein ingestion (P<0.05). *= P<0.05 compared with corresponding fasted value.

Figure 6 Skeletal muscle mRNA expression of MAFBx (A), MuRF1 (B), FOXO1 (C), TNFα (D) and IL-6 (E) in the fasted and fed (4 h following ingestion of 25 g protein) state following 5 days of one-legged knee immobilization in the immobilized (IMMOB) and non-immobilized control (CON) legs of healthy, young men (n=12). Data were analyzed with two-way repeated measures ANOVAs. A: Significant main effect of immobilization (P<0.05). B: Significant main effect of immobilization (P<0.01). 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 compared with corresponding CON values. * = P<0.05 compared with corresponding fasted value.
Figure 7 Muscle phosphorylation status (presented as a ratio of phosphorylated to total protein) of mTOR (A) S6K (B), RS6 (C) and 4-EBP1 (D) in the fasted and fed (4 h following ingestion of 25 g protein) state following 5 days of one-legged knee immobilization in the immobilized (IMMOB) and non-immobilized control (CON) legs of healthy, young men (n=12). Data were analyzed with two-way repeated measures ANOVAs. A: Significant interaction effect (P<0.01). B: No significant effects. C: Significant main effect of feeding (P<0.01). D: Significant interaction effect (P<0.01). †= P<0.05 compared with corresponding CON values. * = P<0.05 compared with corresponding fasted value.
Table 1. Subjects’ characteristics ($n=12$)

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<tr>
<td>Age (y)</td>
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<tr>
<td>Body mass (kg)</td>
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<td>BMI (kg m$^{-2}$)</td>
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<td>Body fat (% of body mass)</td>
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<td>Average leg lean mass (kg)</td>
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<td>HbA1c (%)</td>
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Values represent means±SEM. HbA1c: glycosylated haemoglobin.
Figure 1

Quadriceps CSA (mm$^2$)

- CON
- IMMOB

PRE
POST

*
Figure 2

- Figure A: Plasma insulin (mU/L)
- Figure B: Plasma phenylalanine (µmol/L)
- Figure C: Plasma leucine (µmol/L)
- Figure D: Plasma tyrosine (µmol/L)
- Figure E: Plasma L-[ring-2H5] phenylanine (MPE)
- Figure F: Plasma L-[1-13C] leucine (MPE)
- Figure G: Plasma L-[1-13C] phenylanine (MPE)
Figure 3

A

Post-absorptive myofibrillar FSR (% h\(^{-1}\))

B

Post-prandial myofibrillar FSR (% h\(^{-1}\))

CON  IMMOB
Figure 4

Delta myofibrillar L-[1-13C] phenylalanine enrichment (MPE)
Figure 5

**LAT1**

A

**PAT1**

B

**SNAT2**

C

**CD98**

D

- Relative mRNA expression
- CÓN, IMMOB
- FASTED, FED

* indicates statistical significance.
Figure 7

(A) mTOR (ser2448) phosphorylation (AU)

(B) S6K1 (Thr389) phosphorylation (AU)

(C) R6S (Ser235/236) phosphorylation (AU)

(D) 4E-BP1 (Thr37/46) phosphorylation (AU)