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RESEARCH ARTICLE

Short-term muscle disuse induces a rapid and sustained decline in daily myofibrillar protein synthesis rates

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[1]*Department of Sport and Health Sciences, College of Life and Environmental Science, University of Exeter, Exeter, United Kingdom;* [2]*Peninsula National Institute for Health Research Clinical Research Facility, College of Medicine and Health, University of Exeter, Exeter, United Kingdom;* [3]*Department of Human Biology, NUTRIM School of Nutrition and Translational Research in Metabolism, Maastricht University Medical Centre, Maastricht, The Netherlands.; and* [4]*Institute of Biomedical and Clinical Science, University of Exeter Medical School, Exeter, Devon, United Kingdom*

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Kilroe SP, Fulford J, Holwerda AM, Jackman SR, Lee BP, Gijsen AP, van Loon LJC, Wall BT. Short-term muscle disuse induces a rapid and sustained decline in daily myofibrillar protein synthesis rates. *Am J Physiol Endocrinol Metab* 318: E117–E130, 2020. First published November 19, 2019; doi:10.1152/ajpendo.00360.2019.—Short-term muscle disuse has been reported to lower both postabsorptive and postprandial myofibrillar protein synthesis rates. This study assessed the impact of disuse on daily myofibrillar protein synthesis rates following short-term (2 and 7 days) muscle disuse under free living conditions. Thirteen healthy young men (age: 20 ± 1 yr; BMI: 23 ± 1 kg/m^{-2}) underwent 7 days of unilateral leg immobilization via a knee brace, with the nonimmobilized leg acting as a control. Four days before immobilization participants ingested 400 mL of 70% deuterated water, with 50-mL doses consumed daily thereafter. Upper leg bilateral MRI scans and muscle biopsies were collected before and after 2 and 7 days of immobilization to determine quadriceps volume and daily myofibrillar protein synthesis rates. Immobilization reduced quadriceps volume in the immobilized leg by 1.7 ± 0.3 and 6.7 ± 0.6% after 2 and 7 days, respectively, with no changes in the control leg. Over the 1-wk immobilization period, myofibrillar protein synthesis rates were 36 ± 4% lower in the immobilized (0.81 ± 0.04%/day) compared with the control (1.26 ± 0.04%/day) leg ($P < 0.001$). Myofibrillar protein synthesis rates in the control leg did not change over time ($P = 0.775$), but in the immobilized leg they were numerically lower during the 0- to 2-day period (16 ± 6%, 1.11 ± 0.09%/day, $P = 0.153$) and were significantly lower during the 2- to 7-day period (44 ± 5%, 0.70 ± 0.06%/day, $P < 0.001$) when compared with the control leg. We conclude that 1 wk of muscle disuse induces a rapid and sustained decline in daily myofibrillar protein synthesis rates in healthy young men.

atrophy; disuse; immobilization; muscle protein synthesis rates; skeletal muscle

INTRODUCTION

The recovery from injury or illness requires otherwise healthy individuals to undergo a period of short-term (≤1 wk) muscle disuse and/or physical inactivity, during which rapid skeletal muscle atrophy and declines in functional and metabolic capacity occur (14, 41, 42) (1, 2). It has also been proposed that the accumulation of such short periods of disuse may contribute to the development of age-related sarcopenia (16, 40) (3, 4). Despite the clinical relevance, the physiological mechanisms responsible for muscle disuse atrophy are yet to be fully elucidated.

A loss of skeletal muscle mass must ultimately be explained by a chronic alteration in muscle protein synthesis and/or breakdown rates. We (42) and others (8, 17–20) have shown that experimental muscle disuse results in a decline in postabsorptive muscle protein synthesis rates without any apparent change in muscle protein breakdown rates (22). More recently, we (42) and others (15) have also reported that disuse brings about a resistance to the anabolic properties of protein ingestion. Consequently, it has been suggested that impairments in postabsorptive and postprandial muscle protein synthesis rates largely explain (uncomplicated) muscle disuse atrophy in humans (32, 33, 44).

To date, data concerning disuse-induced alterations in muscle protein synthesis rates have relied on the continuous infusion of stable isotope-labeled amino acids under laboratory conditions and their subsequent incorporation into serial muscle biopsies to capture hour-by-hour muscle protein synthesis rates at limited time points before, during, or after a disuse intervention. Although this allows the controlled assessment of muscle protein synthesis rates, these measurements can only be made over a few hours before potential tracer recycling (i.e., protein-bound tracers being released back into the precursor pools) becomes a confounding variable. As a result, these measurements capture only a small snapshot of time (e.g., 1–2%) that a volunteer spends undergoing muscle disuse and do not account for the combined effects of all lifestyle factors that may contribute to chronically altered muscle protein synthesis rates (e.g., repeated and varied mixed meal ingestion, whole body and altered physical activity patterns, hormonal and diurnal metabolic fluctuations, etc.). Consequently, it is of importance to establish whether measurements of how disuse affects acute muscle protein synthesis rates translate to daily and free living muscle protein synthesis rates and, therefore, can be quantitatively predictive of muscle disuse atrophy. Moreover, the difficulties in obtaining multiple acute measures of muscle protein synthesis rates within the same individual explain the lack of data fully detailing how muscle protein

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turnover is temporally regulated during short-term disuse. This is of interest since it has been suggested that both the rate of atrophy and the contribution of alterations in muscle protein synthesis rates may differ in the first 2 to 3 days of disuse compared with subsequent time points (2).

Recently, the regular ingestion of deuterated water as a means to measure muscle protein synthesis rates has regained popularity in the field due to its capacity to assess free living muscle protein synthesis rates during an intervention lasting days (5, 23, 29, 45) or weeks (9, 30). This methodology has recently been applied to assess cumulative (25) and temporal (9) changes in free living muscle protein synthesis rates throughout resistance training programs of varying durations but, to date, has only minimally been directed at muscle disuse atrophy research (27, 28). In the present work, we applied oral deuterated water dosing methods to assess the temporal impact of disuse on free living and daily myofibrillar protein synthesis rates during 1 wk of limb immobilization in healthy young men. We hypothesized that disuse would reduce myofibrillar protein synthesis rates within 2 days, and to a greater extent than after 1 wk, and this would primarily explain the observed rate of muscle disuse atrophy.

METHODS

Participants. Thirteen healthy young men (age = 20 ± 1 yr, BMI = 23.4 ± 0.9 kg/m^{-2}) were included in the present study. Only young males were included in the present study since both age (38) and sex (10) can influence the rate of muscle disuse atrophy, and our goal was to maintain a homogeneous population for the current study. Participants attended the laboratory for a routine medical screening and completed a general medical questionnaire to assess their eligibility for participation and to ensure no adverse health conditions were present. Exclusion criteria included a (family) history of deep vein thrombosis/cardiovascular disease, metabolic disorders (e.g., type 2 diabetes), musculoskeletal/orthopedic disorders, a body mass index of >28.5 kg/m^{-2} or <18.5 kg/m^{-2}, participation in a structured resistance training program within 6 mo before the study, any musculoskeletal injury of the legs within 12 mo before the study, use of anticoagulants, and consumption of any nutritional supplement before and during the study. During the screening, participants' height, body mass, and blood pressure were measured, and body composition was also assessed by air displacement plethysmography (BODPOD; Life Measurement, Inc.). All subjects were informed of the nature and possible risks of the experimental procedures before providing written, informed consent. The study was part of a wider study assessing muscle disuse atrophy that was approved by The Sport and Health Science Ethics Committee of the University of Exeter (151021/B/02), in accordance with the guidelines set out in the Declaration of Helsinki. This study was registered as a clinical trial with clinicaltrials.gov (NCT02984332).

Experimental design. A graphical representation of the experimental study design can be seen in Fig. 1. Following successful completion of the screening visit, eligible participants attended the laboratory for five experimental visits across 11 days, and this included a 7-day period of unilateral leg immobilization using a leg brace and ambulation via the use of crutches. To measure daily myofibrillar protein synthesis rates throughout the immobilization period, participants underwent a deuterium oxide dosing protocol (described below). This protocol was designed to achieve and maintain 0.8–1.0% body water deuterium enrichment during the measurement periods, in line with our previous work (25). Participants arrived at the laboratory on *day 5* of the experimental protocol at ~0800 for the first of three experimental test days. During this visit, bilateral muscle biopsies were collected from the *m. vastus lateralis*, and an MRI scan was conducted of both thigh muscles (at ~0830; participants were transported to and from the MRI scanner via a wheelchair to ensure no contraction or weight bearing occurred before/after the MRI scans and biopsies). Thereafter, a 7-day immobilization period was started at ~0900. After 2 and 7 days of immobilization, subjects returned to the laboratory at ~0800 for an identical test day. Muscle biopsies were obtained under local anesthesia, using the percutaneous Bergstrom needle biopsy technique (6), from the *m. vastus lateralis* of both legs ~15 cm above the patella and ~3 cm below the fascia. Immediately following a muscle biopsy the muscle tissue was quickly assessed, and

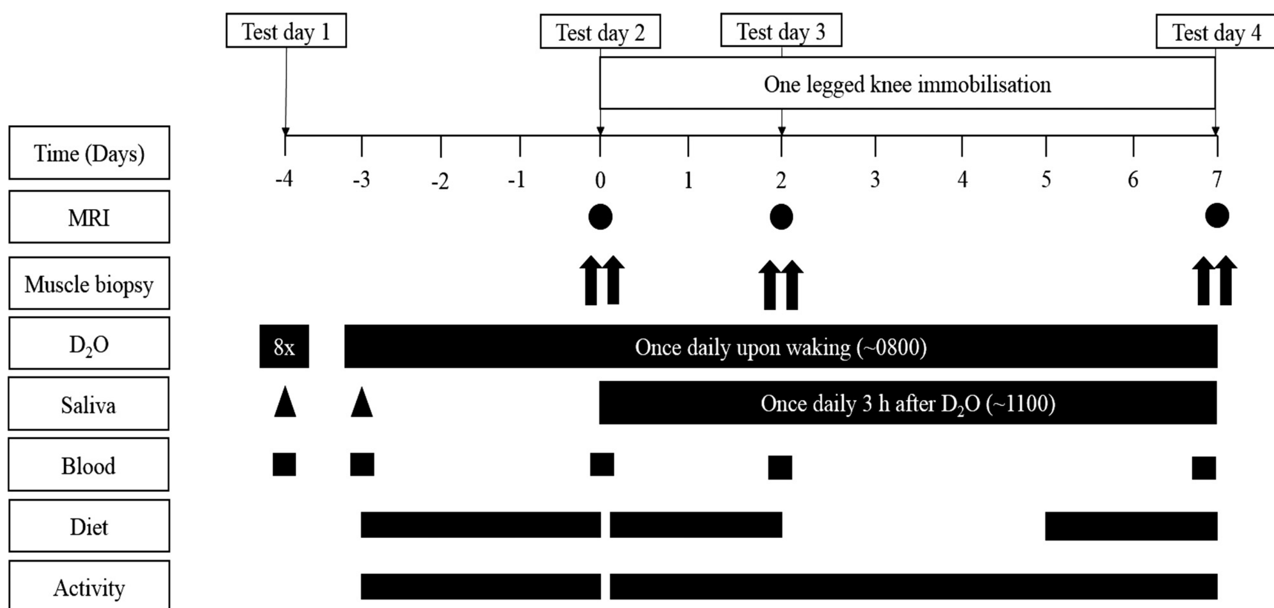

Fig. 1. Study schematic. Thirteen healthy young males underwent 7 days of unilateral leg immobilization via knee brace. Activity is physical activity measured continuously by GENEactiv wrist watch accelerometry. Diet is habitual dietary intake recorded by self-reported, written diet diary. Blood indicates venous blood sample. Arrows represent bilateral m. vastus lateralis muscle biopsies (i.e., taken from the control and immobilized legs). D_2O, deuterated water ingestion; MRI, magnetic resonance imaging.

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any blood or nonmuscle tissue was dissected and discarded. The muscle samples were immediately frozen in liquid nitrogen and stored at −80°C until further analysis.

Immobilization protocol. The model of immobilization used was a unilateral leg brace (X-ACT Donjoy brace; DJO Global, Vista, CA), with the participant ambulating on crutches (after receiving instruction) throughout the immobilization period. The immobilized leg was randomized and counterbalanced for leg dominance, with the nonimmobilized leg acting as a within-subject control. Using the hinge of the brace, the knee was fixed at an angle of 40° flexion (full knee extension = 0°) to ensure that no weight bearing occurred. Subjects were instructed that all ground contact and muscle contraction (except for ankle rotation exercises twice/day to activate the venous muscle pump) in the immobilized leg were forbidden. Adhesive tape with the experimenter's signature inscribed was placed around the straps of the brace. Breaking of the tape would indicate that the brace had been altered and resulted in exclusion from the study (24); no participants were excluded. A plastic shower cover was provided to the participants to wear over the brace when showering. Daily contact was maintained with the subject throughout the study to ensure proper compliance.

Deuterated water dosing protocol. The deuterated water dosing protocol was based on our previous work (25). *Day 1* of the experimental protocol acted as a D_2O loading day, where participants consumed 400 mL of 70% D_2O (i.e., 280 mL of pure D_2O) separated over the day as 8 × 50 mL boluses (CK Isotopes Ltd., Leicestershire, UK). Upon arrival at the laboratory (0730), background blood and saliva samples were collected before the first bolus of D_2O was ingested. The first dose of D_2O was consumed at ~0800, with the remaining doses being consumed every 1.5 h. Participants stayed at the university until four out of the eight loading day D_2O doses had been consumed, with the remaining D_2O doses being consumed at home under instruction of timings (i.e., leaving 1.5 h between each). Every day following the loading day, participants consumed a maintenance dose of D_2O (50 mL) upon waking (~0800 h). One participant reported some mild feelings of vertigo and dizziness during the loading day, which passed after ~2 h. Three hours (~1100) after the daily D_2O maintenance dose, a daily saliva sample was collected using a cotton mouth swab (Celluron, Hartmann, Germany), which the participant lightly chewed for ~1 min until saturated with saliva. The saturated sponge was placed into an empty syringe, where the swab was squeezed to release the saliva into a collection tube. The saliva samples were used to assess the body water ^2H enrichment and stored in the participant's freezer until they were returned at the next study visit. Additional blood samples were collected in the fasted state at the start (i.e., ~0800) of each test day [i.e., *days 5* (pre), *7* (after 2 days of immobilization), and *12* (post)]. Venous blood samples were collected from the antecubital vein via venipuncture technique and collected into EDTA-containing vacutainers that were centrifuged at 4,000 rpm for 10 min at 4°C. Aliquots of plasma were frozen in liquid nitrogen and stored at −80°C until further analysis took place. To ensure uniformity and compliance with the D_2O protocol, participants were provided with a log to record the times they consumed the D_2O and with enough doses to last until their next study visit, at which point containers were returned and counted and subsequent doses provided.

Diet and physical activity. For 3 days before immobilization, subjects' physical activity was measured using an accelerometer (GENEactiv; Activinsights, Cambridgeshire, UK) that was attached to the nondominant wrist. Subjects were instructed to wear the accelerometer continuously, with data being collected at a 60-Hz sampling frequency. Subjects' physical activity was measured again throughout the 7-day immobilization period. Subjects' were instructed to refrain from vigorous physical activity during immobilization but to attempt to maintain their habitual activity levels despite using crutches for ambulation (to avoid whole body sedentariness during immobilization). Physical activity data from the GENEActiv accelerometers were converted into 60-s epochs and used to estimate time spent performing total physical activity (all intensities) using standard cutoff points (11). Subjects' diets were recorded for 3 days (2 weekdays and 1 weekend day) before immobilization by a self-reported written diet diary that followed detailed instructions and advice from a member of the research team. Subjects were asked to refrain from alcohol intake and maintain a similar diet during and throughout the immobilization period, and this was assessed by two further 2-day diet diaries in the first and last 2 days of immobilization; these were averaged to create an "immobilization diet." Dietary analyses for the calculation of energy and macronutrient intakes were completed using specialized nutrition software (Nutritics Professional Nutritional Analysis Software; Swords Co., Dublin, Ireland).

Magnetic resonance imaging and quadriceps volume calculation. MRI scan methodology of the upper legs for the determination of quadriceps volume has been described in detail previously (4). In brief, a 1.5-tesla (T) MRI scanner was used to make axial plane images over the full length of the femur. A T1-weighted three-dimensional turbo spin echo sequence was used (field of view 500 × 500 mm, reconstructed matrix 512 × 512 mm, echo time 15 ms, repetition time 645 ms, slice thickness 5 mm, slice gap 5 mm) with the subject lying still in the supine position; a four-element sense body radiofrequency coil was wrapped around both thighs. Philips online MRI software was used to analyze the images obtained in the axial plane (the same experimenter performed all manual segmentation of the images). Starting at the most distal image (approximately mid-patella) where each muscle group could be fully delineated, the muscle of interest was manually segmented to calculate slice cross-sectional area. Moving proximally, every third image was analyzed until complete delineation of the muscle groups of interest could no longer be identified (the greater trochanter). Muscle volume was calculated using a previously published method (28) where the total CSA for all images was calculated and multiplied by the slice gap plus the distance between slices (linear interpolation; in this case a total of 3 cm, 5-mm slice thickness, 25-mm slice gap), summarized by the following equation:

$$\text{muscle volume} = \sum_{aCSA} \cdot \left(\text{slice thickness} + \text{slice gap}\right).$$

Body water deuterium enrichment. Body water deuterium enrichment was measured using the saliva samples collected daily throughout the course of the study. All saliva samples were centrifuged at 10,000 *g* and subsequently diluted 70-fold with ddH_2O to lower deuterium enrichments so that they were in the detection range of the isotope ratio mass spectrometer (IRMS). Following the dilution step, samples were prepared for analysis on the IRMS using the protocol described by Scrimgeour et al. (34). In summary, small plastic cups holding 4 mg of 5% platinum on alumina, 325 mesh (Sigma-Aldrich, St. Louis, MO), were placed inside 3-ml glass vials (Labco Exetainer; Labco, Lampeter, UK). Subsequently 300 μL of the diluted saliva samples was placed into the vials, and the vials were then sealed using rubber septums and a screw cap. The air within each vial was simultaneously evacuated and replaced by hydrogen gas. Vials were then left for 24 h at 21°C for deuterium equilibration between the hydrogen gas and saliva samples to occur. The deuterium enrichment of the hydrogen gas was then measured in duplicate on an IRMS (Micromass Optima IRMS fitted with a multiprep and Gilson autoinjector; Micromass, Manchester, UK). 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 deuterium loss during equilibration.

Plasma free [^2H]alanine enrichments. Plasma amino acid enrichments were determined by gas chromatography-mass spectrometry (GC-MS) analysis (5975C MSD and 7890A GC; Agilent, Wilmington, DE). First, the plasma samples were deproteinized using dry 5-sulfosalicylic acid. Subsequently, free amino acids were purified using cation exchange chromatography (AG 50W-X8 resin, mesh size

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100–200 μm, ionic form: hydrogen; Bio-Rad Laboratories, Hercules, CA). The purified amino acids were converted to their *tert*-butyldimethylsilyl (*tert*-BDMS) derivatives with MTBSTFA before analysis via GC-MS analysis. The plasma free alanine mass isotopomers (M and M + 1) were measured using selective ion monitoring at *m/z* 232 and 233. 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.

Myofibrillar bound ^2H alanine enrichments. Myofibrillar protein-enriched fraction was extracted from ~50 mg of wet weight muscle tissue by hand homogenization on ice using a pestle in a standard extraction buffer (10 μL/mg). The samples were centrifuged at 2,500 *g* for 5 min at 4°C, and the pellet was then washed with 500 μL of ddH2O and again centrifuged at 2,500 *g* for 10 min at 4°C. The myofibrillar protein was solubilized by adding 1 ml of 0.3 M NaOH and heating for 30 min at 50°C, with samples being vortexed every 10 min. Samples were then centrifuged for 10 min at 9,500 *g* and 4°C, the supernatant containing the myofibrillar protein was kept, and the collagen protein pellet was discarded. The myofibrillar proteins were precipitated by the addition of 1 mL of 1 M PCA and spun at 700 *g* and 4°C for 10 min. Myofibrillar proteins were then washed with 70% ethanol twice and hydrolyzed overnight in 2 mL of 6 M HCl at 110°C. The free amino acids from the hydrolyzed myofibrillar protein pellet were dried under a nitrogen stream while being heated at 120°C. The free amino acids were subsequently dissolved in 25% acetic acid solution and passed over cation exchange AG 50W-X8 resin columns (mesh size: 100–200; ionic form: hydrogen; Bio-Rad Laboratories, Hercules, CA) and eluted with 2 M NH_4OH. Following this, the eluted amino acids were dried and the purified amino acids derivatized to their N(O,S)-ethoxycarbonyl ethyl esters (26). The derivatized were measured using a gas chromatograph isotope ratio mass spectrometer (GC-IRMS, MAT 253; Thermo Fisher Scientific, Bremen, Germany) equipped with a pyrolysis oven and a 60m DB-17MS column (no. 122-4762; Agilent) and a 5-m precolumn. Ion masses 2 and 3 were analyzed to determine the $^2H/^1H$ ratios of muscle protein-bound alanine. A series of known standards was used to assess the linearity of the mass spectrometer and to control for the loss of tracer.

Skeletal muscle mRNA analyses. Skeletal muscle mRNA expression of 48 genes involved in the regulation of skeletal muscle mass and metabolism were analyzed (gene names and symbols are listed in Table 1) as described previously (11). In brief, total RNA was isolated from ~20 mg of frozen muscle tissue using TRIzol Reagent (Life Technologies, Invitrogen) according to the supplier's instructions. Total RNA was quantified spectrophotometrically at 260 nm, and RNA purity was assessed as the ratio of readings at 260/280 nm (Nanodrop ND-1000 Spectrophotometer; Thermo Fisher Scientific). Subsequently, first-strand cDNA was synthesized from 150 ng of RNA using a SuperScript III cDNA synthesis kit (cat. no. 11752-050; Invitrogen, Life Technologies). Taqman low-density, custom-designed array cards (ABI Applied Biosystems, Foster City, CA) were used for the relative quantification of expression of the 48 selected genes. Each card ran eight samples simultaneously against 48 Taqman gene expression assay probes that were prefixed into each well on the card. In summary, 50 μL of Taqman universal master mix (ABI; Applied Biosystems) was added to 150 ng of RNA equivalent cDNA in an RNase-free Eppendorf, with RNase free water being added to a make a final reaction volume of 100 μL. Samples were vortexed and centrifuged (briefly) and then pipetted into sample reservoirs on the micro fluidic array cards; following this the array cards were centrifuged (1,000 rpm for 1 min, Hereaus 3 S-R Microfuge; Thermo Fisher Scientific, Waltham, MA) and then run on a QuantStudio 12K Flex Real-Time PCR system (ABI; Applied Biosystems). Relative quantification of the genes was performed using the $\Delta\Delta C_T$ method ($2^{-\Delta\Delta C_T}$), and data were normalized to the geometric mean of *GAPDH* and *18s* genes (42).

Table 1. *Names and symbols of the gene expression assay targets preloaded upon PCR microfluidic cards*

Gene Name (Human Skeletal Muscle)	Symbol
Insulin receptor substrate 1	*IRS1*
Phosphoinositide-3-kinase regulatory subunit 1	*PI3K*
AKT serine/threonine kinase 2	*AKT2*
Mechanistic target of rapamycin	*mTOR*
Ribosomal protein S6 kinase B2	*RPS6KB1 (P70s6k)*
Eukaryotic translation initiation factor 4E binding protein 1	*EIF4EBP1 (4E-BP1)*
Solute carrier family 7 member 5	*SLC7A5 (LAT1)*
Solute carrier family 38 member 2	*SLC38A2 (SNAT2)*
Solute carrier family 36 member 1	*SLC36A1 (PAT1)*
DNA damage inducible transcript 4	*DDIT4 (REDD1)*
Eukaryotic 18S rRNA	*18s rRNA*
Forkhead box O1	*FOXO1*
Forkhead box O3	*FOXO3*
Calpain 1	*CLPN1*
Calpain 3	*CLPN3*
Caspase 3	*CASP3*
Proteasome subunit-β1	*PSMB1*
Nuclear factor-κB subunit 1	*NFKB*
F-box protein 32	*MAFbx*
Tripartite motif containing 63	*TRIM63 (MuRF1)*
Beclin 1	*BECN1*
Myostatin	*MTSN*
Growth differentiation factor 11	*GDF11*
Solute carrier family 2 member 4	*GLUT4*
Glycogen Phosphorylase (muscle)	*PYGM*
Glycogen synthase 1	*GS1*
Hexokinase 2	*HK2*
Pyruvate dehydrogenase kinase 4	*PDK4*
CD36 molecule	*CD36*
Fatty acid synthase	*FASN*
Sterol regulatory element binding transcription factor 1	*SHREBP1*
Carnitine palmitoyltransferase 1B	*CPT1B*
Acetyl-CoA carboxylase-β	*ACACA*
Peroxisome proliferator activated receptor-α	*PPARa*
PPARG coactivator-1α	*PGC1a*
Mitofusin 1	*MFN1*
Dynamin 1 like protein	*DNM1L*
Transcription factor A, mitochondrial	*TFAM*
Dystrophin	*DMD*
Sarcoglycan-α	*SGCA*
Laminin subunit-α2	*LAMA2*
Integrin subunit-β1	*ITGB1*
Actinin-α3	*ACTN1*
Desmin	*DES*
Vascular endothelial growth factor A	*VEGF*
Hypoxia inducible factor 1 alpha subunit	*HIF-1*
Angiopoietin 1	*ANGPT1*
Glyceraldehyde-3-phosphate dehydrogenase	*GAPDH*

Symbols in parentheses represent gene synonyms.

Calculations. Myofibrillar protein fractional synthesis rates (FSR) were calculated based on the incorporation of [^{2}H]alanine into myofibrillar protein and either the mean free plasma [^{2}H]alanine or the mean body water deuterium enrichment throughout the time period as precursors. FSR was calculated using the standard precursor-product method expressed as daily rates as follows:

$$\mathrm{FSR}(\% \cdot \mathrm{day}^{-1}) = \left(\frac{E_{m2} - E_{m1}}{E_{\mathrm{precursor}} \times t}\right) \times 100,$$

where E_{m1} and E_{m2} are the myofibrillar muscle protein-bound enrichments on either *days 0* and *2*, *0* and *7*, or *2* and *7*, respectively. $E_{\mathrm{precursor}}$ represents either mean plasma free [^{2}H]alanine or mean body water deuterium enrichment corrected by a factor of 3.7 based on deuterium labeling of alanine during de novo synthesis (mean enrich-

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ment between *days 0* and *2*, *0* and *7*, or *2* and *7*) (15, 24); *t* represents the time between biopsies (*days 0* and *2*, *0* and *7*, or *2* and *7*).

Statistics. All data are presented as means ± SE, and all statistical analyses were conducted in GraphPad Prism version 7.0 (GraphPad Software, San Diego, CA). A paired-samples *t*-test was used to compare myofibrillar protein synthesis rates in the control versus immobilized legs between *days 0* and *7* and physical activity and dietary intake between pre-immobilization and during immobilization. Two-way repeated-measures ANOVAs with leg (immobilized and control) and time [*days 0–2* and *2–7* (for myofibrillar protein synthesis rates) and days 0, 2 and 7 (for MRI)] as within-subjects factors were used to compare differences in myofibrillar protein synthesis rates over time, gene expression, and quadriceps volume. Pearson's correlation coefficient was used to assess the relationship between tracer precursor pools. For all ANOVAs, when a significant interaction was found, Bonferroni post hoc tests were applied to locate individual differences. Statistical significance was set at $P < 0.05$.

RESULTS

Diet and physical activity analyses. Table 2 shows participants' habitual dietary intake and physical activity data averaged for 3 days preceding and for 4 (for dietary intake) and 7 days (for physical activity) during the immobilization period. No differences in energy or macronutrient intake (fat, carbohydrate, and protein) were observed between pre-immobilization and during immobilization (all $P > 0.05$). Light and vigorous physical activity were not different between pre-immobilization and during immobilization ($P > 0.05$). Moderate physical activity reduced from pre-immobilization to during immobilization ($P = 0.032$).

Quadriceps muscle volume. Quadriceps muscle volumes of the control and immobilized legs were determined by MRI (previously reported in detail in Ref. 27a). There were no differences in quadriceps volume between legs pre-immobilization [control leg = 2,315 ± 120 cm^3, immobilized leg = 2,342 ± 129 cm^3 ($P = 0.993$)], and the control leg remained unchanged throughout the study ($P = 0.981$). However, a significant time × leg interaction was detected ($P < 0.001$) such that quadriceps volume of the immobilized leg had decreased by 1.7 ± 0.3% after 2 days (to 2,301 ± 127 cm^3, $P = 0.007$) and by a further 5.0 ± 0.6% between *days 2* and *7* (to 2,186 ± 117 cm^3, $P < 0.001$), resulting in a total decrease in quadriceps muscle volume of 6.7 ± 0.6% ($P < 0.001$) over the full week of immobilization.

Table 2. *Dietary intake and physical activity levels during a habitual period and during 1 wk of single leg immobilization*

	Pre-Immobilization	During Immobilization
Energy intake, MJ/day (kcal/day)	11.1 ± 0.7 (2,647 ± 167)	11.3 ± 1.0 (2,689 ± 288)
Protein intake		
g/day	119 ± 7	115 ± 12
$g \cdot kg^{-1} \cdot day^{-1}$	1.6 ± 0.1	1.6 ± 0.1
En%	18 ± 1	17.3 ± 1
Carbohydrate intake		
g/day^{-1}	298 ± 25	308 ± 42
En%	45 ± 2	46 ± 2
Fat intake		
g/day	102 ± 10	108 ± 11
En%	35 ± 2	36 ± 2
Light physical activity, h/day	1.4 ± 0.2	1.0 ± 0.1
Moderate physical activity, h/day	2.6 ± 0.4	1.7 ± 0.2*
Vigorous physical activity, h/day	0.3 ± 0.1	0.1 ± 0.1
Total physical activity, h/day	4.3 ± 0.5	2.8 ± 0.2*

Values represent means ± SE; $n = 13$. *Significant difference from pre-immobilization value, $P < 0.05$.

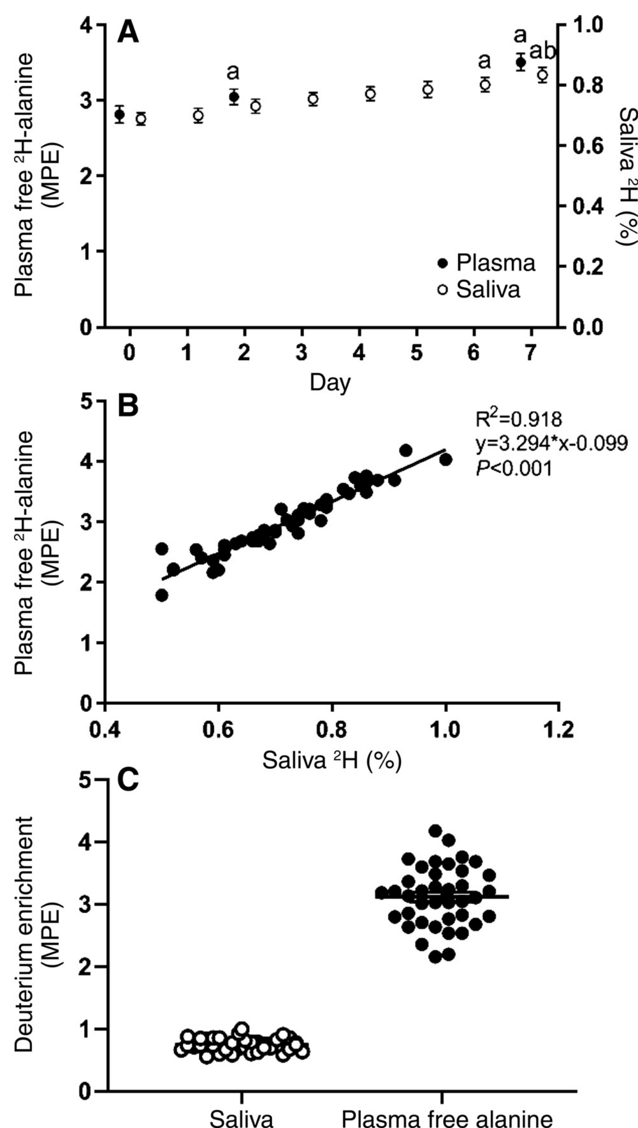

Fig. 2. Stable isotope precursor pool data. *A* and *B*: plasma free [^{2}H]alanine [mole percent excess (MPE); *left y*-axis] and saliva ^{2}H (%; *right y*-axis) enrichments displayed over the week of immobilization (*A*), with data analyzed using 1-way repeated measures ANOVAs, and the correlation between saliva ^{2}H (%) and plasma free [^{2}H]alanine (MPE) analyzed by a Pearson's correlation analysis (*B*). *C*: comparison of individual participant saliva enrichment (%) and plasma free [^{2}H]alanine (MPE) enrichments at the *day 0*, *2*, and *7* time points. [a]Significant difference from *day 0* in corresponding precursor pool, $P < 0.05$; [b]significant difference from *day 1* in saliva precursor pool, $P < 0.05$. Data are means ± SE; $n = 13$.

Precursor pool enrichments. Saliva deuterium enrichments (Fig. 2*A*) reached 0.69 ± 0.02% on *day 0* (start of immobilization) and averaged 0.71 ± 0.02, 0.78 ± 0.03, and 0.76 ± 0.02% over 0–2, 2–7, and 0–7 time points, respectively. Saliva deuterium enrichments showed a modest increase over the immobilization period (main effect of time, $P < 0.001$) such

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that enrichments on *days 6* (0.80 ± 0.02%, $P = 0.024$) and *7* (0.83 ± 0.03%, $P < 0.001$) were elevated above *day 0*, and *day 7* enrichments were higher than *day 1* (0.70 ± 0.02%, $P = 0.003$). Plasma free [^{2}H]alanine enrichments (Fig. 2*A*) reached 2.8 ± 0.1 mole percent excess (MPE) on *day 0* and averaged 3.1 ± 0.2 MPE over the week of immobilization. Plasma free [^{2}H]alanine enrichments also showed a slight increase over the immobilization period (main effect of time, $P < 0.001$) such that *day 2* (3.1 ± 0.1 MPE, $P = 0.017$) and *day 7* (3.5 ± 0.1 MPE, $P < 0.001$) enrichments were higher than *day 0* (2.8 ± 0.1 MPE). A Pearson's correlation coefficient showed that the saliva deuterium and the plasma [^{2}H]alanine precursor pools were strongly correlated (data from all time points, 0–2, 2–7, and 0–7, collapsed into 1 test, $r = 0.959$, $P < 0.001$; Fig. 2*B*). Plasma free [^{2}H]alanine enrichments were on average 3.93-fold greater than saliva deuterium enrichments (Fig. 2*C*). It should be acknowledged that we have not determined muscle intracellular free [^{2}H]alanine enrichments in the present work. It is conceivable that our intervention may have influenced global muscle alanine metabolism divergently across legs, which could affect local precursor enrichments. However, our (27) and others' (9) previous observations that muscle intracellular free [^{2}H]alanine does not differ substantially across legs even when unilateral leg interventions are applied (e.g., daily resistance-type exercise) and correlate tightly with plasma free [^{2}H]alanine enrichments suggest the latter to be a valid precursor pool.

Daily myofibrillar protein synthesis rates. Myofibrillar protein-bound [^{2}H]alanine enrichments showed significant time ($P < 0.001$), leg ($P < 0.001$), and time × leg interaction ($P < 0.001$) effects. After 2 days of immobilization, myofibrillar protein bound [^{2}H]alanine enrichments increased by 20 ± 10% more in the control (0.0695 ± 0.0045 MPE) compared with the immobilized (0.0579 ± 0.0042 MPE) leg ($P = 0.511$). After 7 days, myofibrillar-bound protein [^{2}H]alanine enrichments increased over time in both legs but by 53 ± 11% more in the control (0.2448 ± 0.0096 MPE) compared with the immobilized (0.1596 ± 0.0079 MPE) leg ($P < 0.001$). Daily myofibrillar FSRs (%/day) were calculated using mean (of each individual's data during that time period) saliva deuterium enrichments (corrected by a factor of 3.7 to account for the labeling coefficient between saliva and plasma; see Refs. 15 and 25) and then separately using mean plasma free [^{2}H]alanine enrichments (again, an individual's own data averaged over the period of interest) as precursor pools over the 0–2, 2–7, and 0–7 time points of immobilization (Fig. 3). Over the entire week of immobilization, daily myofibrillar FSR, calculated using either the saliva deuterium enrichment (control leg = 1.26 ± 0.05%/day and immobilized leg = 0.81 ± 0.04%/day) or plasma free [^{2}H]alanine enrichment (control leg = 1.14 ± 0.05%/day; immobilized leg = 0.71 ± 0.04%/day) as the precursor pools were 36 ± 4 ($P < 0.001$) and 37 ± 4% ($P < 0.001$) lower in the immobilized compared with the control leg, respectively. A Pearson's *r* product moment correlation showed a strong correlation between FSR calculated from the two precursor pools, with data collapsed for both legs over the full week of immobilization ($r = 0.982$, $P < 0.001$; Fig. 3*C*).

When assessing the temporal changes in myofibrillar FSR over the early (i.e., 0–2 days) and late (i.e., 2–7 days) immo-

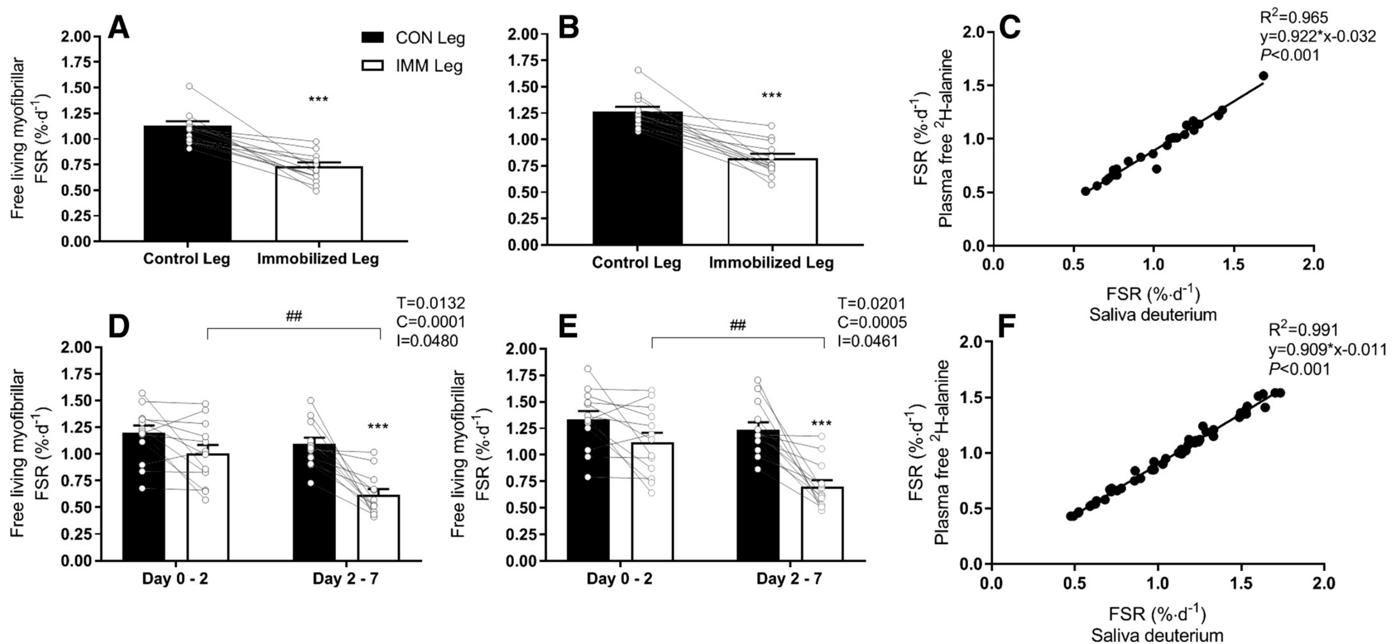

Fig. 3. Free living myofibrillar fractional synthesis rates (%/day). *A* and *B*: free living myofibrillar fractional synthesis rates (FSR; %/day) over the full week calculated from plasma and saliva precursor pools, respectively, as individually assessed by paired-samples *t*-test. *D* and *E*: free living myofibrillar FSR between *days 0* and *2* and *2* and *7* of immobilization calculated from plasma and saliva precursor pools, respectively, as assessed by 2-way repeated measures ANOVA (leg × time), with time (*T*), condition (C), and interaction (I) effects displayed above each graph. Where a significant time × treatment interaction was detected, Bonferroni post hoc tests were conducted to locate individual differences; ***$P < 0.001$, significant difference between legs at the corresponding time point; ##$P < 0.01$ significant difference within leg between the *day 0* and *2* and *2* and *7* time points. *C* and *F*: correlations between myofibrillar FSRs calculated from the 2 different precursors between *days 0–7* (*C*) and *days 0–2* and *2–7* combined (*F*), analyzed by Pearson's correlation analyses. Data are means ± SE for the noncorrelation graphs; $n = 13$.

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bilization periods and using the saliva deuterium enrichment precursor, significant main effects of time ($P = 0.020$), condition ($P < 0.001$), and a time × condition interaction ($P = 0.046$) were detected. Specifically, myofibrillar FSR was 16 ± 6 ($P = 0.153$) and 44 ± 5% ($P < 0.001$) lower at 0–2 (control leg = 1.32 ± 0.08%/day; immobilized leg = 1.11 ± 0.09%/day) and 2–7 days (control leg = 1.24 ± 0.07%/day; immobilized leg = 0.70 ± 0.06%/day) in the immobilized compared with the control leg. Myofibrillar FSR remained unchanged over time in the control leg but declined by 37 ± 11% from 0 to 2 to 2 to 7 days ($P = 0.005$) in the immobilized leg. When using the plasma free [^{2}H]alanine enrichment to assess temporal changes in myofibrillar FSR over the early (i.e., 0–2 days) and late (i.e., 2–7 days) immobilization periods, significant main effects of time ($P = 0.013$), condition ($P < 0.001$), and a time × condition interaction ($P = 0.048$) were detected. Specifically, myofibrillar FSR was 16 ± 6 ($P = 0.159$) and 44 ± 5% ($P < 0.001$) lower at 0–2 (control leg = 1.19 ± 0.07%/day; immobilized leg = 1.01 ± 0.08%/day) and 2–7 days (control leg = 1.11 ± 0.07%/day; immobilized leg = 0.62 ± 0.05%/day) in the immobilized compared with the control leg. Myofibrillar FSR remained unchanged over time in the control leg but declined by 38 ± 11% from 0 to 2 to 2 to 7 days ($P = 0.003$) in the immobilized leg. A Pearson's r product moment correlation showed a strong correlation between the two precursor pools for both legs over both the 0- to 2- and 2- to 7-day time points (both legs and time periods collapsed into 1 test, $r = 0.995$, $P < 0.001$; Fig. 3*F*).

Skeletal muscle gene expression. The skeletal muscle expression of genes involved in insulin signaling and muscle protein synthesis/growth factors, muscle protein breakdown, carbohydrate and lipid metabolism, and mitochondrial metabolism/angiogenesis/force transduction are shown in Figs. 4, 5, 6, and 7, respectively. The remaining genes of the 46 analyzed are shown in Fig. 8. Thirteen of the analyzed genes (*P70S6K*, *EIF-4EBP1*, *REDD1*, *MTSN*, *GS1*, *HK2*, *FAT*, *CPT1B*, *PPARa*, *DNML1*, *TFAM*, *SCGA*, and *ANGPT1*) showed no changes throughout the experiment (time, condition, and interaction effects all $P > 0.05$). Twenty genes exhibited a time effect (all $P < 0.05$) only such that they changed to an equivalent extent

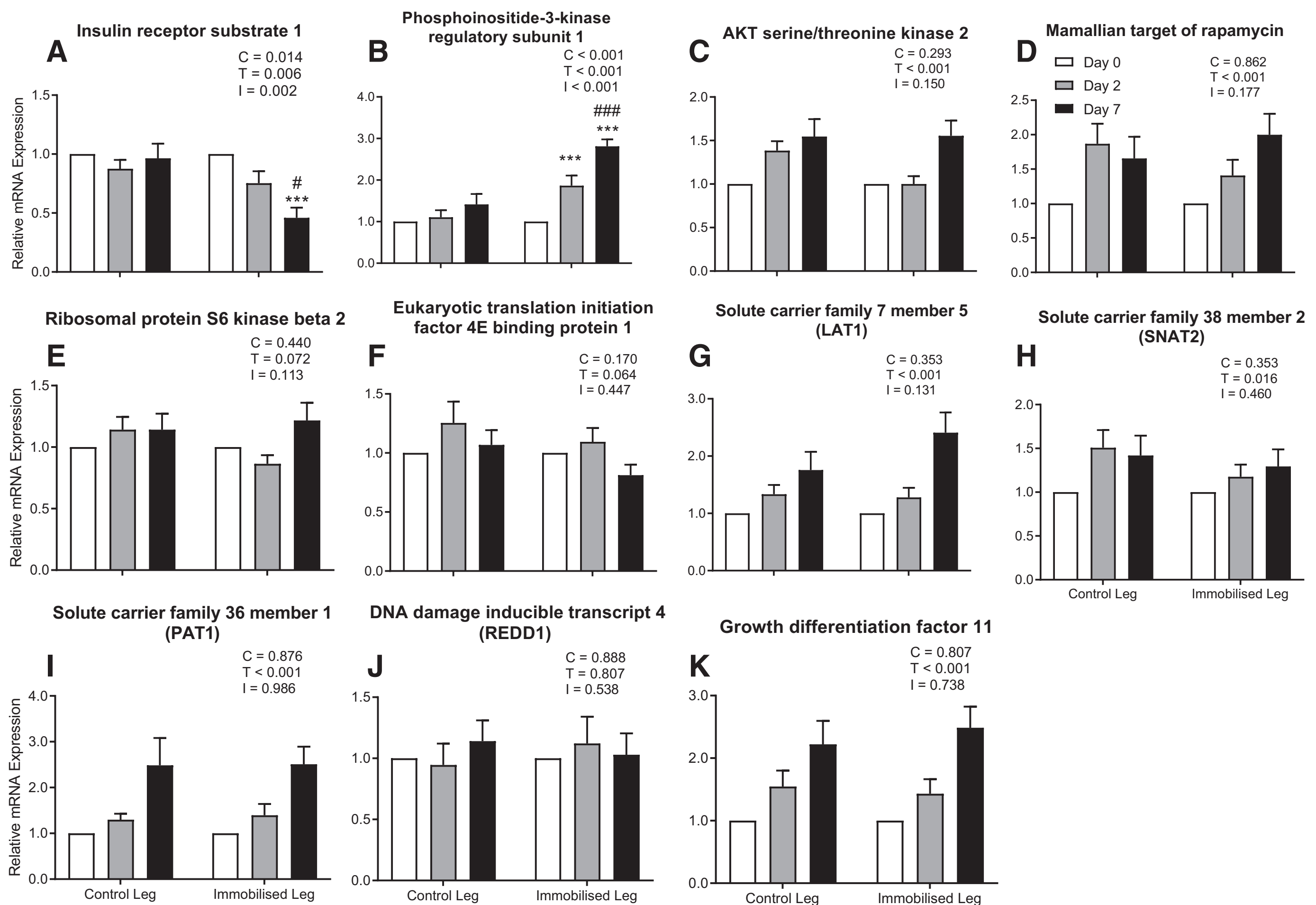

Fig. 4. Skeletal muscle mRNA expression of genes involved in muscle protein synthesis and/or identified as growth factors in healthy young males before and following 2 and 7 days of unilateral leg immobilization in the control and immobilized legs. Data from each gene were analyzed using a 2-way repeated-measures ANOVA (leg × time), and time (*T*), condition (C; i.e., leg), and interaction (I) effects are displayed above each graph. Where a significant interaction was found, Bonferroni post hoc tests were applied to locate individual differences. ***$P < 0.001$, significant differences from *day 0* within the same leg; #$P < 0.05$ and ###$P < 0.001$, significant differences from *day 2* within the same leg. Relative quantification of the genes was performed using the $\Delta\Delta C_T$ method ($2^{-\Delta\Delta C_T}$), with data normalized to the geometric mean of 2 housekeeping genes (*GAPDH* and *18s*) and pre-immobilization (i.e., *day 0*, normalized to a value of 1) in both the control and immobilized legs. Data are means ± SE; $n = 13$.

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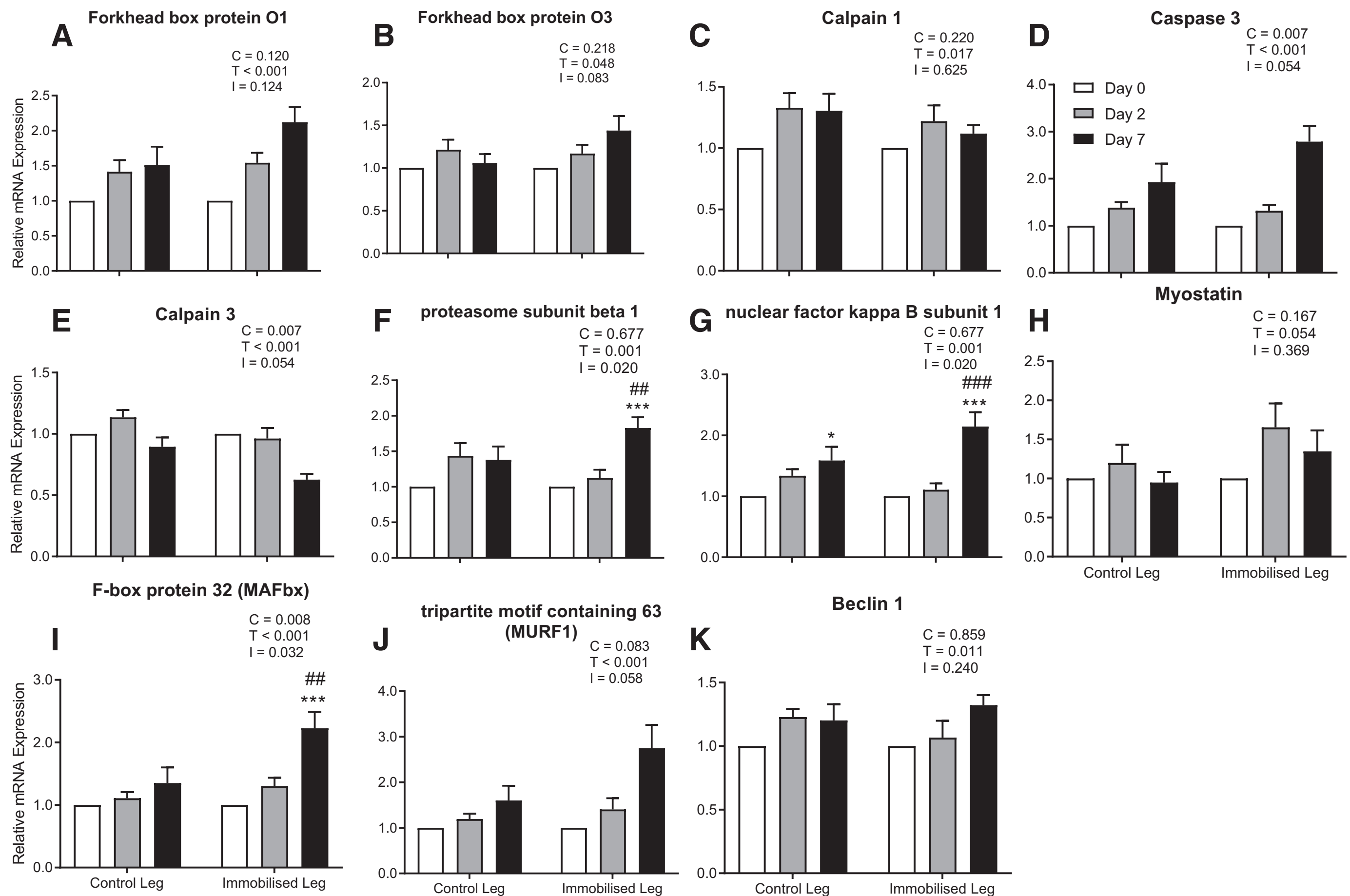

Fig. 5. Skeletal muscle mRNA expression of genes involved in muscle protein breakdown in healthy young males before and following 2 and 7 days of unilateral leg immobilization in the control and immobilized legs. Data from each gene were analyzed using a 2-way repeated measures ANOVA (leg × time), and time (*T*), condition (C; i.e., leg), and interaction (I) effects are displayed above each graph. Where a significant interaction was found, Bonferroni post hoc tests were applied to locate individual differences. $^*P < 0.05$ and $^{***}P < 0.001$, significant differences from *day 0* within the same leg, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$, significant differences from *day 2* within the same leg. Relative quantification of the genes was performed using the $\Delta\Delta C_T$ method ($2^{-\Delta\Delta C_T}$), with data normalized to the geometric mean of 2 housekeeping genes (*GAPDH* and *18s*) and pre-immobilization (i.e., *day 0*, normalized to a value of 1) in both the control and immobilized legs. Data are means ± SE; $n = 13$.

[either an increase (*AKT2*, *LAT1*, *PAT1*, *CLPN1*, *GDF11*, *FASN*, *MFN1*, *ITGB1*, *HIF1a*, *DES*, *mTOR*, *SHREBP1*, *BECN1*, *PDK4*, *FOXO1* and *SNAT2*) or a decrease (*PYGM*)] in both the control and immobilized legs. Some of these genes (*ACABa*, *DMD*, and *VEGFa*) also exhibited a treatment effect (all $P < 0.05$) without any interaction (all $P > 0.05$). Thirteen genes (*MAFbx*, *PGC1a*, *PSMB1*, *NFKb*, *IRS1*, *ACTN3*, *PI3K*, *MuRF1*, *FOXO3*, *CASP3*, *CD36*, *CLPN3*, and *GLUT4*) displayed an interaction effect such that divergent responses occurred between legs over time (all $P < 0.05$). With one exception (*PI3K*), these responses were restricted to 7 but not 2 days of immobilization. For example, after 7 days of immobilization the relative expression of *PSMB1* (Fig. 6*F*) and *MAFBx* (Fig. 6*I*) increased by 83 ± 15 and 123 ± 26% (both $P < 0.001$), respectively, in the immobilized leg only. *NFKb* (Fig. 6*G*) increased in both legs at 7 days but to a greater extent in the immobilized compared with the control leg (115 ± 24 vs. 59 ± 23%; $P = 0.027$). Muscle mRNA expression of *IRS1* (Fig. 5*A*), *ACNT3* (Fig. 8*I*), and *PGC1a* (Fig. 8*B*) remained unchanged in the control leg ($P > 0.05$) but reduced by 54 ± 9, 77 ± 7, and 52 ± 6%, respectively, in the immobilized leg after 7 days (all $P < 0.001$). Only *PI3K* (Fig. 5*B*) expression increased after both 2 (87 ± 24%, $P < 0.001$) and 7 (181 ± 17%, $P < 0.001$) days in the immobilized leg, with no change in the control leg ($P = 0.774$). *MuRF1* (Fig. 6*J*), *FOXO3* (Fig. 6*B*), *CASP3* (Fig. 6*D*), *CD36* (Fig. 7*E*), and *CLPN3* (Fig. 6*E*) all displayed trends for a divergent response in relative expression between legs over the immobilization period (interaction effects all $P < 0.10$). The relative expression of *MuRF1* and *FOXO3* did not change after 2 days but displayed a trend to increase by 175 ± 51 and 112 ± 22%, respectively, and *CLPN3* a trend to decrease by 37 ± 5%, after 7 days in the immobilized leg (all $P < 0.05$), with no changes in the control leg (all $P > 0.05$). *CASP3* increased in both legs at 7 days but to a greater extent in the immobilized compared with control leg (179 ± 34 vs. 92 ± 40%, $P = 0.313$). Muscle *CD36* expression displayed a trend to increase in the control leg after 2 (73 ± 51%) and 7 (65 ± 49%) days, with no change in the immobilized leg ($P = 0.994$).

DISCUSSION

In the current study, we applied a deuterated water approach and performed MRI scans to assess the temporal

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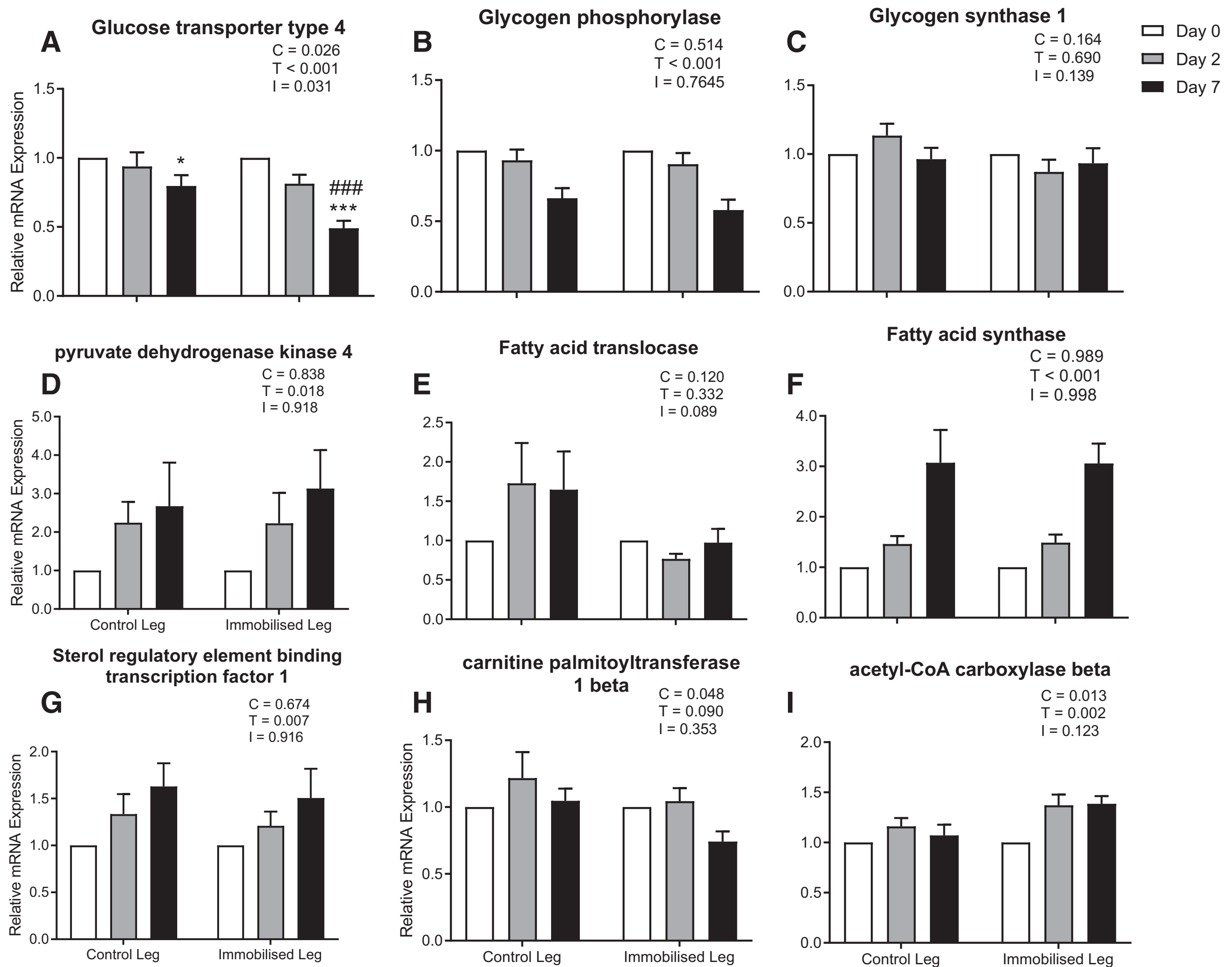

Fig. 6. Skeletal muscle mRNA expression of genes involved in carbohydrate and/or lipid metabolism in healthy young males before and following 2 and 7 days of unilateral leg immobilization in the control and immobilized legs. Data from each gene were analyzed using a 2-way repeated-measures ANOVA (leg × time), and time (*T*), condition (C; i.e., leg), and interaction (I) effects are displayed above each graph. Where a significant interaction was found, Bonferroni post hoc tests were applied to locate individual differences. $*P < 0.05$ and $***P < 0.001$, significant differences from *day 0* within the same leg; $\#\#\#P < 0.001$, significant differences from day 2 within the same leg. Relative quantification of the genes was performed using the $\Delta\Delta C_T$ method ($2^{-\Delta\Delta C_T}$), with data normalized to the geometric mean of 2 housekeeping genes (*GAPDH* and *18s*) and pre-immobilization (i.e., *day 0*, normalized to a value of 1) in both the control and immobilized legs. Data are means ± SE; $n = 13$.

impact of 1 wk of unilateral leg immobilization on daily free living cumulative myofibrillar protein synthesis rates and quadriceps muscle volume, respectively. We report several novel observations. First, immobilization lowered daily free living myofibrillar protein synthesis rates by 36% (compared with the control leg) over 1 wk of immobilization, and the magnitude of this decline was positively correlated with the observed decline in quadriceps volume. Second, this decline in myofibrillar protein synthesis rates occurred rapidly within 2 days, but to a greater extent in the latter part (2–7 days) of the week of immobilization. Finally, 1 wk, but not 2 days, of muscle disuse was associated with a coordinated upregulation in the muscle expression of genes involved in the ubiquitin proteasome-mediated muscle protein breakdown pathway.

Skeletal muscle loss during a period of disuse has been attributed to a decline in both basal (postabsorptive) (8, 20, 42) and postprandial muscle protein synthesis rates (13, 42). Currently, there are fewer data available on the impact of disuse on muscle protein synthesis rates throughout the day, which includes basal, postprandial, and overnight muscle protein synthesis rates. To gain more insight into the impact of disuse on daily muscle protein synthesis rates >2 and ≤7 days of immobilization, we applied the use of deuterated water. The ingestion of deuterated water has been applied to assess muscle protein synthesis rates in response to acute (27) and longer-term (9, 31) resistance training as well as in response to various nutritional interventions (31). Recently, other groups have used the provision of deuterated water to measure the effect of disuse on muscle protein synthesis rates (27, 28). In line with previous work from our group(s) (24, 35), we show that our oral deuterated water regimen resulted in a sustained elevation in body water deuterium (~0.76 ± 0.02%; Fig. 2*A*) and plasma deuterated alanine (~3.1 ± 0.2 MPE; Fig. 2*A*) enrich-

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Fig. 7. Skeletal muscle mRNA expression of genes involved in carbohydrate and/or lipid metabolism in healthy young males before and following 2 and 7 days of unilateral leg immobilization in the control and immobilized legs. Data from each gene were analyzed using a 2-way repeated measures ANOVA (leg × time), and time (*T*), condition (C; i.e., leg), and interaction (I) effects are displayed above each graph. Where a significant interaction was found, Bonferroni post hoc tests were applied to locate individual differences. $^{**}P < 0.01$ and $^{***}P < 0.001$, significant differences from *day 0* within the same leg; ##$P < 0.01$ and ###$P < 0.001$, significant differences from *day 2* within the same leg. Relative quantification of the genes was performed using the $\Delta\Delta C_T$ method ($2^{-\Delta\Delta C_T}$), with data normalized to the geometric mean of 2 housekeeping genes (*GAPDH* and *18s*) and pre-immobilization (i.e., *day 0*, normalized to a value of 1) in both the control and immobilized legs. Data are means ± SE; $n = 13$.

ments throughout the experimental period. The data from both precursor pools also correlated tightly (Fig. 2*B*) and quantitatively related to one another, in agreement with previous work (Fig. 2*C*) (27). Combined with the unilateral immobilization approach (a within-subject comparison of muscle contraction status with identical precursor pool supply), we were able to calculate that disuse reduced free living daily myofibrillar protein synthesis rates by ~36% over 1 wk of immobilization

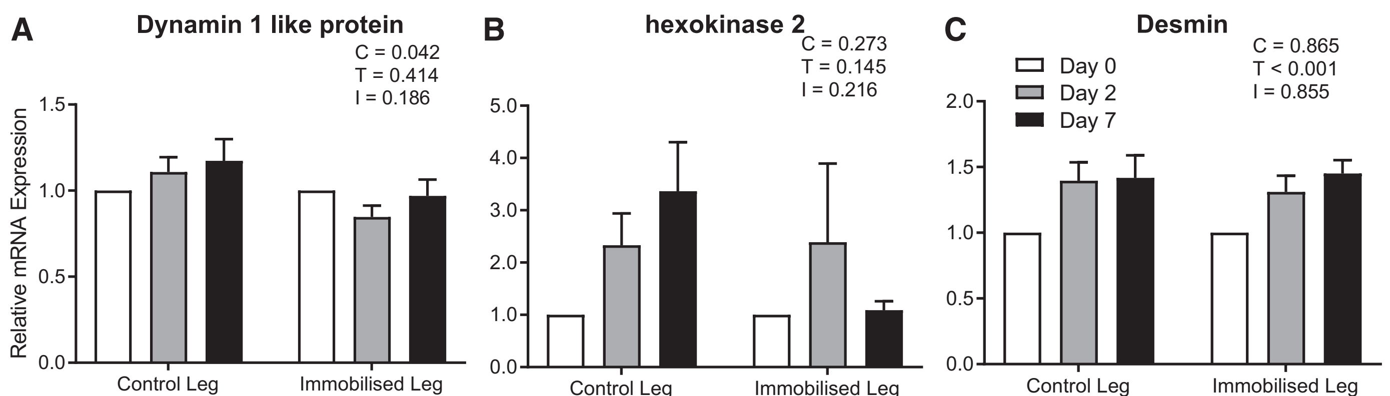

Fig. 8. Skeletal muscle mRNA expression of dynamin-like protein 1 (*A*), desmin (*B*), and hexokinase 2 (*C*) in healthy young males before and following 2 and 7 days of unilateral leg immobilization in the control and immobilized legs. Data from each gene were analyzed using a 2-way repeated-measures ANOVA (leg × time), and time (*T*), condition (C; i.e., leg), and interaction (I) effects are displayed above each graph. Relative quantification of the genes was performed using the $\Delta\Delta C_T$ method ($2^{-\Delta\Delta C_T}$), with data normalized to the geometric mean of 2 housekeeping genes (*GAPDH* and *18s*) and pre-immobilization (i.e., *day 0*, normalized to a value of 1) in both the control and immobilized legs. Data are means ± SE; $n = 13$.

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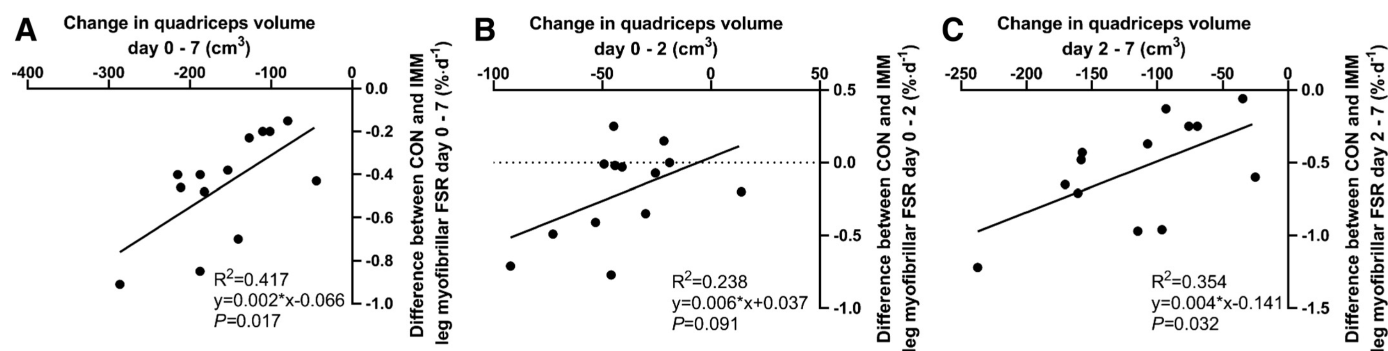

Fig. 9. Correlations between the change in quadriceps volume in the immobilized (IMM) leg and the difference between control (CON) and IMM leg myofibrillar fractional synthesis rates (FSR) across the following time points: *days 0–7* (*A*), *days 0–2* (*B*), and *days 2–7* (*C*). Data analyzed by Pearson's correlation analyses, r^2 and *P* values displayed on each graph; $n = 13$.

(Fig. 3*B*). This reduction is quantitatively in line with what would be predicted from previous studies that have assessed the effects of 5 to 14 days of leg immobilization on myofibrillar protein synthesis rates using both stable isotope-labeled amino acid tracers (8, 22, 44) and deuterium oxide approaches (29). Moreover, we also report that the individual decline in myofibrillar protein synthesis rates was strongly positively correlated with the amount of muscle tissue lost during the week of disuse (Fig. 9*A*). Thus, we provide robust evidence to show that a chronic (i.e., presumably for 24 h/day) decline in daily myofibrillar protein synthesis rates plays a major role in driving muscle disuse atrophy in vivo in humans. Worthy of note is that this decline occurred despite our volunteers maintaining a relatively high dietary protein intake of 1.6 $g \cdot kg^{-1} \cdot day^{-1}$ (see Table 2), suggesting that the impairments were exclusively a result of the disuse per se and not further augmented by any compensatory decrease in protein (or energy) intake. In addition, the decline in synthetic rate was observed within the myofibrillar fraction of the muscle protein pool, underlining that targeting the maintenance (or stimulation) of myofibrillar protein synthesis rates during short-term disuse should clearly represent a primary strategy to combat the loss of muscle mass and associated declines in function (11, 12, 27, 43).

A clear picture of how muscle protein synthesis rates change over time during a period of disuse is not yet available, primarily because of the technical difficulties associated with making multiple acute measurements of myofibrillar protein synthesis rates within the same person (21). As a result, it is not clear how quickly myofibrillar protein synthesis rates decline consequent with disuse or whether the magnitude of decline is dependent on the duration of disuse. Experiments utilizing static molecular markers within muscle tissue have suggested that muscle disuse atrophy may be differentially regulated within the first few days compared with ≥1 wk (1, 37). In the present work, we show that although the decline in MPS rates did not reach statistical significance over the first 2 days, the daily decline in myofibrillar protein synthesis rates during this period [i.e., 8%/day (or 16% in total); Fig. 3, *D* and *E*] was of a magnitude similar to the daily decline observed over *days 2–7* [i.e., 9%/day (or 45% in total); Fig. 3, *D* and *E*]. Clearly, the decline in muscle protein synthesis rates as a major factor in driving muscle loss (Fig. 3, *D* and *E*) is a process that occurs rapidly and seems to be a key process in explaining the fast decline in quadriceps volume that we observed during the first 2 days. However, although the daily rate of decline was similar across the two time periods, the absolute decline in myofibrillar protein synthesis rates consequently occurred to a much greater extent during the latter phase of the disuse period (i.e., 2–7 days). This could suggest that the effect of disuse is cumulative with time, at least for a few days. Alternatively, these data may indicate that an immediate drop in myofibrillar protein synthesis rates occurs but is variable in the time it takes to manifest across individuals (11 of 13 subjects decline after 2 days and 13 of 13 decline after 7 days). Of note is that the tight correlation of declining myofibrillar protein synthesis rates with rate of muscle atrophy was less clear when the early phase of disuse was examined (Fig. 4). Therefore, it is interesting to speculate to what extent declines in myofibrillar protein synthesis rates may quantitatively explain the observed muscle atrophy.

Previous work using data obtained from acute measurements of hourly muscle protein synthesis rates has estimated that reductions in muscle protein synthesis rates can fully explain (32) or even overexplain (22) observed muscle atrophy after 3–6 wk of leg immobilization. In contrast, we have previously estimated from our work that reductions in basal and/or postprandial muscle protein synthesis rates could explain ~80% of the muscle atrophy seen during 5–14 days of disuse (44). However, these estimations rely on a number of assumptions, most importantly that measuring myofibrillar protein synthesis rates after a period of disuse reflects the changes in chronic myofibrillar protein synthesis rates that occur throughout the entire period of disuse and usually inferring muscle mass from single slice cross-sectional measurements. In the present study, we have captured a cumulative myofibrillar protein synthetic response throughout the entire period of disuse alongside the calculation of quadriceps mass. If, as has previously been done (32), we assume that muscle protein breakdown rates remained unchanged with disuse and assume in the control leg that daily MPB rates were equivalent to daily MPS rates, we can calculate the expected loss of muscle mass as a result of decreased daily MPS rates as follows: net muscle protein loss = daily MPB – daily MPS. Using this approach, we calculate a net muscle protein loss of 0.21 and 0.45%/day over the first 2 days and entire week, respectively. Given that we know the muscle mass of the participants' quadriceps at baseline, this equates to an expected muscle protein loss from the immobilized quadriceps of 11 and 76 g over 2 and 7 days, respectively, that is directly attributable to reduced MPS rates. Surprisingly, this

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accounts for only 25 and 47% of the 42 and 162 g of estimated (measured) muscle loss. Despite improving the resolution of such calculations, our methods still involve a number of assumptions to arrive at these figures, most notably that muscle density does not differ substantially across volunteers or in response to disuse, that vastus lateralis muscle protein turnover rates are analogous to the entire quadriceps mass, that alanine synthesis rates are representative of other (and all) amino acids, that myofibrillar protein atrophy is equivalent to total protein loss, and that the absence of a "true precursor pool" (i.e., labeled tRNA) does not considerably alter the absolute rates arrived at. Because it is likely that such limitations induce some error in the calculated contribution of muscle protein synthesis to muscle atrophy, indirect conclusions on the reciprocal role of muscle protein breakdown are difficult to make with confidence. However, the fact that we can account for only (less than) one-half of the observed muscle atrophy due to impaired MPS rates is surprising and not in line with previous work (21, 32). Consequently, it is intriguing for future work to endeavor to make accurate and direct measures of muscle protein breakdown in humans to establish any (temporal) causative role in human muscle disuse atrophy. This is an elusive but highly consequential physiological question that was beyond the scope of the present work.

With the difficulty of measuring in vivo MPB rates within the constraints of the current design, we applied a custom-designed, low-density RT-quantitative PCR microarray gene card to measure the relative expression of 46 targeted genes that encode proteins associated with the regulation of muscle mass, muscle deconditioning/reconditioning, and muscle metabolism, with a specific sub-focus on genes involved in MPB. Thirteen genes were unaffected, with a further 20 showing an equivalent change in both legs (likely due to a repeated biopsy effect; see Ref. 39). Thirteen of our selected genes changed differentially in the immobilized compared with control leg, and a striking observation is that seven of those genes are involved in muscle protein breakdown, four of which encode proteins involved in the ubiquitin proteasome system [UPS; *PSMB1* (Fig. 5*F*), *MAFbx* (Fig. 5*I*), and *MuRF1* (Fig. 5*J*)] (7) and associated transcription factors (e.g., *FOXO3*; see Fig. 5*B*). This coordinated change in gene expression of components of the UPS typically manifested as an increase after 7 but not 2 days of immobilization. In contrast, genes involved in other muscle protein breakdown pathways, for example, the calcium-dependent calpain system (3), were either unaffected (*CLPN1*; see Fig. 5) or tended to be reduced after 7 but not 2 days of immobilization (*CLPN3*; Fig. 5). These findings are line with and extend on our (42) and other research groups' (27) previous findings that genes specifically involved in the ubiquitination and degradation of myofibrillar proteins show a transcriptional rise consequent with disuse. Previous work has suggested that such a rise in gene expression is not immediate (11) but rapid (between 2 and 7 days) and transient (typically subsiding at time points beyond 1–2 wk) and possibly indicative of an increased rate of muscle protein breakdown and/or metabolic deconditioning during this early phase (37, 42, 44). With respect to the latter, the expression of various genes involved in insulin-mediated glucose uptake (e.g., *IRS1* and *GLUT4*) also declined after 7 but not 2 days of immobilization, interestingly at time points known to succeed the physiological manifestation of insulin resistance (13, 36, 46). Therefore, collectively, our gene array data point to widespread muscle deconditioning stretching beyond solely a loss of muscle mass. A limitation of the present study is that only men were included. Although this allowed for a more homogenous population to allow us to precisely measure and describe temporal, muscle-specific disuse atrophy, it prevents the data from being generalizable to females. Given that sex-based differences in the rate of muscle disuse atrophy likely exist (10), it is of importance that future work assesses whether similar results are demonstrable in women.

In conclusion, muscle disuse induces a rapid decline in muscle volume (within 2 days) that continues at a similar rate for 1 wk. The decline in muscle volume is accompanied by an ~36% decline in daily myofibrillar protein synthesis rates in healthy young men over 1 wk of disuse. These data highlight the key responsible role that declining myofibrillar protein synthesis rates play in the development of muscle disuse atrophy in vivo in humans.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

S.P.K., L.J.v.L., and B.T.W. conceived and designed research; S.P.K., J.F., A.M.H., S.R.J., B.P.L., A.P.G., and B.T.W. performed experiments; S.P.K. and B.T.W. analyzed data; S.P.K., L.J.v.L., and B.T.W. interpreted results of experiments; S.P.K. and B.T.W. prepared figures; S.P.K. and B.T.W. drafted manuscript; S.P.K., L.J.v.L., and B.T.W. edited and revised manuscript; S.P.K., J.F., A.M.H., S.R.J., B.P.L., A.P.G., L.J.v.L., and B.T.W. approved final version of manuscript.

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