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RESEARCH ARTICLE

Daily resistance-type exercise stimulates muscle protein synthesis in vivo in young men

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[1]*NUTRIM School of Nutrition and Translational Research in Metabolism, Maastricht University Medical Centre+ (MUMC+), Maastricht, The Netherlands; and* [2]*Stable Isotope Research Center (SIRC), Maastricht University Medical Center+ (MUMC+), Maastricht, The Netherlands*

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Holwerda AM, Paulussen KJ, Overkamp M, Smeets JS, Gijsen AP, Goessens JP, Verdijk LB, van Loon LJ. Daily resistance-type exercise stimulates muscle protein synthesis in vivo in young men. *J Appl Physiol* 124: 66–75, 2018. First published September 21, 2017; doi:10.1152/japplphysiol.00610.2017.— Resistance-type exercise increases muscle protein synthesis rates during acute postexercise recovery. The impact of resistance-type exercise training on (local) muscle protein synthesis rates under free-living conditions on a day-to-day basis remains unclear. We determined the impact of daily unilateral resistance-type exercise on local myofibrillar protein synthesis rates during a 3-day period. Twelve healthy young men (22 ± 1 yr) were recruited to participate in this study where they performed daily, unilateral resistance-type exercise during a 3-day intervention period. Two days before the exercise training subjects ingested 400 ml deuterated water (^2H_2O). Additional 50-ml doses of deuterated water were ingested daily during the training period. Saliva and blood samples were collected daily to assess body water and amino acid precursor deuterium enrichments, respectively. Muscle tissue biopsies were collected before and after the 3 days of unilateral resistance-type exercise training from both the exercised and the nonexercised, control leg for the assessment of muscle protein synthesis rates. Deuterated water dosing resulted in a steady-state body water enrichment of 0.70 ± 0.03%. Intramuscular free [^{2}H]alanine enrichment increased up to 1.84 ± 0.06 mole percent excess (MPE) before the exercise training and did not change in both the exercised and control leg during the 3 subsequent exercise training days (2.11 ± 0.11 and 2.19 ± 0.12 MPE, respectively; $P > 0.05$). Muscle protein synthesis rates averaged 1.984 ± 0.118 and 1.642 ± 0.089%/day in the exercised vs. nonexercised, control leg when assessed over the entire 3-day period ($P < 0.05$). Daily resistance-type exercise stimulates (local) muscle protein synthesis in vivo in humans.

NEW & NOTEWORTHY This study demonstrates that daily resistance-type exercise stimulates muscle protein synthesis rates in vivo in humans over multiple days. Whereas acute studies have shown that resistance-type exercise increases muscle protein synthesis rates by 50–100%, we observed a lower impact of resistance-type exercise under free-living conditions. We also compared precursor tracer selection for the calculation of muscle protein synthesis rates and observed that saliva deuterium enrichment serves as an appropriate and practical choice of precursor.

deuterated water; exercise; heavy water; muscle protein synthesis

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INTRODUCTION

Skeletal muscle mass is regulated by the net balance between muscle protein synthesis and breakdown rates, which typically show a turnover rate of 1–2% per day (28). Physical activity and food ingestion represent two major anabolic stimuli that increase muscle protein synthesis rates. A single session of resistance-type exercise increases muscle protein turnover (6, 15, 42) and sensitizes skeletal muscle tissue to the anabolic properties of protein ingestion (14, 37, 41). Protein ingestion increases plasma amino acid availability, which further augments the acute postexercise increase in muscle protein synthesis rates (18, 19, 40, 53, 57). Whereas the synergistic effects of resistance-type exercise and protein ingestion have been well established in acute postexercise settings, it remains to be established how such acute findings align with more long-term anabolic responses to exercise.

Contemporary stable amino acid infusion techniques combined with muscle biopsy sampling allows for acute in vivo assessment of muscle protein synthesis rates in humans (10, 47, 55). However, subjects are restricted to the laboratory environment during amino acid tracer infusions and are typically tested under rested and fasted conditions to ensure a high level of control during the laboratory assessment. Furthermore, acute infusion protocols may lose reliability after more than 12 h due to "recycling" of the infused amino acid tracer between protein and precursor pools (50, 54). Consequently, it has been debated to what extent the acute postexercise muscle protein synthetic response to a single bout of exercise can be predictive of the absolute changes in skeletal muscle mass that can be observed during more long-term exercise training interventions (3, 33, 36).

Recently, oral deuterated water (^2H_2O) dosing has re-emerged in the field as a method to assess muscle protein synthesis rates over a period of days or even weeks (22, 49). Ingestion of deuterated water enriches the body water pool with deuterium, allowing for endogenous deuterium labeling of the nonessential amino acid alanine. The deuterium-labeled alanine is synthesized into muscle proteins, which can be detected in muscle biopsy samples and used to calculate muscle protein synthesis rates (48, 49, 61). Test participants are able to remain in their free-living environment, providing the opportunity to incorporate important anabolic factors such as dietary protein intake, habitual physical activity, exercise, hormonal responses, and diurnal variations into the assessment

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Table 1. *Subjects' baseline characteristics of healthy young men who completed unilateral resistance-type exercise on 3 consecutive experimental test days*

Age, yr	22 ± 1
Weight, kg	77.7 ± 2.7
Height, cm	180 ± 2
BMI, kg/m^2	23.9 ± 0.5
LBM, kg	63.2 ± 1.9
Body fat, %	16 ± 1
Unilateral leg extension 1RM, kg	72 ± 4
Unilateral leg press 1RM, kg	133 ± 7
Protein intake, g·kg^{-1}·day^{-1}	1.7 ± 0.2
Step count, steps/day	7,266 ± 686

Values are expressed as means ± SE; $n = 12$. BMI, body mass index; LBM, lean body mass; 1RM, one repetition maximum.

of daily muscle protein synthesis rates. For example, Wilkinson et al. and Brooks et al. have recently applied the deuterated water method in humans to assess temporal patterns of the muscle anabolic response to resistance-type exercise training over 8 days (61) and 6 wk (8), respectively. As such, the deuterated water method appears to represent a valuable tool to further increase our understanding of the many factors that contribute to the regulation of muscle protein turnover and, as such, may even serve to better predict changes in skeletal muscle mass following pharmaceutical, nutritional and/or exercise interventions.

Here, we applied oral deuterated water-dosing methods to determine the impact of resistance-type exercise training on local muscle protein synthesis rates over a three-day period. To test our hypothesis that resistance-type exercise training increases muscle protein synthesis rates over multiple days, we recruited 12 young men (21 ± 1 y) for testing. All subjects performed unilateral resistance-type exercise every day during a three-day intervention period. Subjects ingested small amounts of deuterated water each day throughout the experiment to enrich the body water pool. Saliva and blood samples were collected to assess body water deuterium and plasma free [^{2}H]alanine enrichments, respectively. Muscle tissue biopsies were collected before and after the 3 days of unilateral resistance-type exercise training from the exercised as well as the nonexercised, control leg for the assessment of local muscle protein synthesis rates.

MATERIALS AND METHODS

Test participants. A total of 12 healthy young men (22 ± 1 yr and 23.9 ± 0.5 kg/m^2) were recruited to participate in the present study. Subject characteristics are presented in Table 1. All subjects were screened for inclusion by filling in a medical questionnaire. Upon inclusion, all subjects were performing activities of daily living but were not participating in any structured exercise program totaling more than 4 h of exercise per week. All subjects were informed of the nature and possible risks of the experimental procedures before their written informed consent was obtained. The study was approved by the Medical Ethical Committee of the Maastricht University Medical Centre, The Netherlands (METC 15-3-008) and conformed to standards for the use of human subjects in research as outlined in the most recent version of the Declaration of Helsinki. The study was registered at Netherlands Trial Register as NTR5099.

Pretesting. During the initial screening visit, subjects were familiarized with the study exercise program and had their maximal strength [one repetition maximum (1RM)] estimated on the exercise machines. Subjects first performed a 5-min cycling exercise warm-up at 150 W before completing an estimation of their 1RM on the leg press and leg extension exercises using the multiple repetitions testing procedure (32). Subjects completed the strength testing with the leg (dominant or nondominant) that was randomly chosen to complete the exercise program during the study protocol. For each exercise machine, subjects performed 10 submaximal or warm-up repetitions to become familiarized with the equipment and to have lifting technique critiqued and corrected. Subjects then performed sets at progressively increasing loads until failing to complete a valid repetition, judged by their inability to complete the full range of motion for an exercise. Ideally, subjects failed within three to six repetitions during the last and heaviest set. A 2-min resting period between subsequent attempts was allowed. The pretesting and experimental trials were separated by a period of at least 7 days.

Experimental protocol. A graphical representation of the study design is presented in Fig. 1. The study protocol was designed to assess how daily resistance-type exercise training impacts muscle protein synthesis rates over multiple days while participating in normal daily physical activities and consuming a normal diet. The study protocol consisted of 7 consecutive test days. To measure mixed muscle protein synthesis rates over multiple days, subjects underwent a deuterium oxide-dosing protocol. The deuterated water-dosing protocol (described below) was initiated on *day 2* and was continued over the last 6 days of the experimental protocol. The dosing protocol was designed to achieve and maintain 0.8–1.0% body water deuterium enrichment between *days 3* and *7*. Subjects reported to the laboratory at 1000 on test *day 4* to have one muscle biopsy collected from each leg. Following the muscle biopsies, subjects completed the first of three unilateral resistance-type exercise sessions (described below). After completing each exercise session, test subjects ingested 30 g whey protein dissolved in water with an added 1.5 ml vanilla flavor. Subjects then left the laboratory and were instructed to maintain their normal routine but refrain from any exhaustive physical activity. Subjects returned to the laboratory between 0900 and 1100 on *days 5* and *6* to complete the final two exercise sessions. Subjects returned to the laboratory at 1000 on *day 7* when the final muscle biopsies were collected from each leg signifying the end of the research protocol.

Deuterated water-dosing protocol. The deuterated water-dosing protocol consisted of 1 dosing day and 5 maintenance days. The dosing protocol was modified from previously published studies that have administered deuterated water in human subjects (39, 48, 49). On

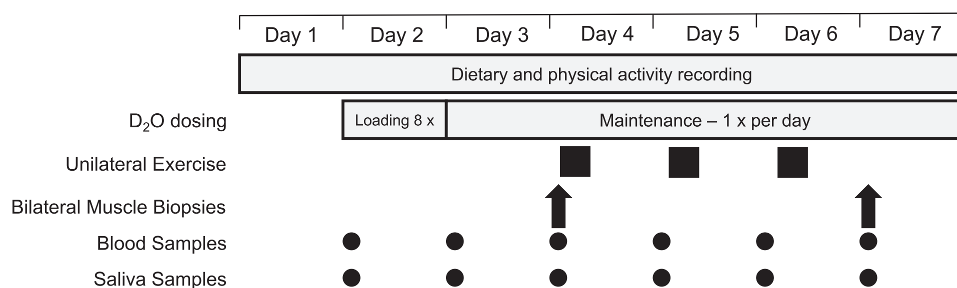

Fig. 1. Graphical representation of the experimental protocol.

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the second test day, subjects reported to the laboratory to initiate the deuterated water-dosing protocol. On the dosing day, subjects ingested 400 ml of 70% deuterium oxide (Cambridge Isotopes Laboratories, Andover, MA) spaced evenly over the day (8 × 50 ml). Upon arrival at 0800, background blood and saliva samples were collected before ingestion of the first 50-ml dose of 70% deuterium oxide. After the first dose, body weight was measured using a scale and body composition and bone mineral content were measured by dual-energy X-ray absorptiometry (DEXA) scan (Discovery A; Hologic, Bedford, MA). Subjects remained in the laboratory until the fifth of eight doses was ingested and were then sent home with the remaining doses of the entire protocol. Subjects were instructed to ingest the three remaining doses of the dosing day at home with 1.5 h in between. Subjects ingested one dose at or before 0800 for each subsequent test day. Although dizziness or vertigo has been previously reported (39, 44), no subjects reported any side effects of deuterated water dosing. Subjects reported to the laboratory each of the subsequent test days to have blood and saliva collected (*days 3–7*), for muscle biopsies (*days 4* and *7*) and for the unilateral resistance-type exercise sessions (*days 4*, *5*, and *6*).

Blood samples were collected in EDTA-containing tubes and centrifuged at 1,000 *g* for 10 min at 4°C. Aliquots of plasma were frozen in liquid nitrogen and stored at −80°C. Saliva samples were collected at least 30 min after meal ingestion. To collect saliva, subjects lightly chewed on a cotton swab (Celluron, Hartmann, Germany) for enough time to saturate the cotton swab with saliva. The swab was then removed and depressed using a syringe to extract the saliva into a sample tube. After collection, saliva was frozen in liquid nitrogen and stored at –80°C. Muscle biopsies were obtained from the middle region of the medialis vastus lateralis, 15 cm above the patella and ~4 cm below entry through the fascia, using the percutaneous needle biopsy technique (5). Muscle biopsy samples were dissected carefully and freed from any visible nonmuscle material. The muscle samples were immediately frozen in liquid nitrogen and stored at –80°C until further analysis.

Diet and physical activity. All subjects were instructed to refrain from any exhaustive physical activity and to keep their diet as consistent as possible 72 h before the trial. Starting on the first test day, subjects began recording their diet and physical activity in diaries and continued to do so for the remaining test days. Step count was assessed during the experiment using an accelerometer (Actical; Philips Respironics). Upon arrival to the laboratory for the deuterated water-dosing day (*day 2*), subjects started wearing the accelerometer on their right hip at the mid clavicular line for the duration of the experiment.

Resistance-type exercise sessions. The resistance-type exercise sessions consisted of 45 min of moderate-intensity lower-body resistance-type exercise. Subjects exercised with a single leg, which was randomized based on dominance (i.e., dominant vs nondominant). Subjects first completed 10 min of unilateral cycling at 75 W with a cadence of 60–80 rpm. Afterwards, subjects performed 1 warm-up set at 50% 1RM followed by 4 sets of 8–10 repetitions at 75% 1RM on the horizontal leg press machine (Technogym, Rotterdam, The Netherlands). After the leg press exercise was completed, subjects performed 1 warm-up set at 50% 1RM followed by 4 sets of 8–10 repetitions at 75% 1RM on the leg extension machine (Technogym). Subjects were allowed to rest for 2 min between all sets.

Body water deuterium enrichment. Body water enrichment was analyzed using the saliva samples collected throughout the experimental protocol. All samples were centrifuged at 10,000 *g* to remove any debris. Following centrifugation, all samples were diluted 70-fold with ddH_2O to achieve deuterium enrichments within the detection limits of the gas chromatograph-combustion-isotope ratio mass spectrometer (GC-C-IRMS). After dilution, samples were prepared for analysis on a GC-C-IRMS using the protocol outlined by Scrimgeour et al. (51). Briefly, small plastic cups holding 4 mg of catalyst (5% platinum on alumina, 325 mesh; Sigma-Aldrich, St. Louis, MO) were placed inside 3-ml glass vials (Labco Exetainer; Labco, Lampeter, UK). Three-hundred microliters of diluted saliva sample were then transferred into the vials. The glass vials were sealed using rubber septums and a screw cap. Air in each vial was simultaneously evacuated and replaced by hydrogen gas. The prepared vials were left at 21°C for 24 h for deuterium equilibration to occur between the hydrogen gas and the saliva samples. The deuterium enrichment of the hydrogen gas was then measured in duplicate on a GC-C-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 [^{2}H]alanine enrichment. Plasma amino acid enrichments were determined by gas chromatography-mass spectrometry analysis (GC-MS; Agilent 5975C MSD & 7890A GC, Wilmington, DE). Plasma samples were deproteinized with dry 5-sulfosalicylic acid. Free amino acids were purified using cation exchange chromatography (AG 50W-X8 resin, mesh size: 100–200 μm, ionic form: hydrogen; Bio-Rad Laboratories, Hercules, CA). The purified amino acids were converted into *tert*-butyldimethylsilyl (*tert*-BDMS) derivatives with MTBSTFA before analysis by GC-MS. The plasma free alanine mass isotopomers (M and M + 1) were determined 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.

Intramuscular free and bound [^{2}H]alanine enrichment. For measurement of [^{2}H]alanine enrichment in the intramuscular free and mixed muscle protein pools, 55 mg wet muscle were freeze dried. Collagen, blood, and other nonmuscle fiber material were removed from the muscle fibers under a light microscope. The isolated muscle fiber mass (2–3 mg) was weighed, and 7 vol (7 times dry weight of isolated muscle fibers wet:dry ratio) of ice-cold 2% perchloric acid were added. The tissue was then sonicated and centrifuged. The supernatant was collected and processed in the same manner as the plasma samples, such that intracellular free [^{2}H]alanine enrichments could be measured by using their *t*-butyldimethylsilyl derivatives on a GC-MS. The protein pellet was washed with three additional 1.5-ml washes of 2% perchloric acid, dried, and hydrolyzed in 6 M HCl at 120°C for 15–18 h. The hydrolyzed protein fraction was dried under a nitrogen stream while being heated at 120°C. After being dissolved with a 50% acetic acid solution, samples were passed over Dowex exchange resin (AG 50W-X8, 100–200 mesh hydrogen form; Bio-Rad, Hercules, CA) by using 2 M NH_4OH. Thereafter, the eluate was dried, and the purified amino acids were derivatized to their N(O,S)-ethoxycarbonyl ethyl esters (27). The derivatized samples were measured using a gas chromatography-isotope ratio mass spectrometer (MAT 253; Thermo Fisher Scientific, Bremen, Germany) equipped with a pyrolysis oven using a 60-m DB-17MS column and 5-m precolumn (No. 122–4762; Agilent) and GC-Isolink. Ion masses 2 and 3 were monitored to determine the ^{2}H/^{1}H ratios of muscle protein bound alanine. A series of known standards were applied to assess linearity of the mass spectrometer and to control for the loss of tracer.

Western blotting. Muscle was homogenized as previously described (56). After protein quantification, equal amounts (50 μg protein/lane) of protein were loaded and standard SDS-PAGE procedures were followed. Antibodies included total and phosphorylated mammalian target of rapamycinm (mTOR; Ser^{2448}), S6 protein kinase 1 (S6K1; Thr^{389}), RS6 (Ser^{235}/Ser^{236}), anti-phospho-eukaryotic translation initiation factor 4E-binding protein-1 (4E-BP1; Thr$^{37/46}$), anti-mTOR, anti-S6K1, anti-ribosomal protein S6 (RS6), and anti-4E-BP1, which were purchased from Cell Signaling Technology (Danvers, MA). α-Tubulin (Abcam) was used as a loading control. All samples for a given protein were detected on the same membrane using chemiluminescence and the FluorChem HD imaging system (Alpha Innotech, Santa Clara, CA).

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Calculations. Mixed muscle protein fractional synthetic rate (FSR) was determined using the incorporation of [^{2}H]alanine into muscle proteins and either mean intracellular free [^{2}H]alanine enrichment in each leg, mean free [^{2}H]alanine enrichment in plasma, or mean body water deuterium enrichment corrected by a factor of 3.7 based on the deuterium labeling during de novo alanine synthesis. FSR was calculated using the standard precursor-product method:

$$\mathrm{FSR}\left(\%/\mathrm{day}^{-1}\right) = \left(\frac{E_{m2} - E_{m1}}{E_{\mathrm{precursor}} \quad xt}\right) \times 100\% \qquad (1)$$

where E_{m1} and E_{m2} are the mixed muscle protein-bound enrichments on *days 4* and *7*, respectively; $E_{\mathrm{precursor}}$ represents either mean intracellular free [^{2}H]alanine enrichment in each leg, mean free [^{2}H]alanine enrichment in plasma, or body water deuterium enrichment corrected by a factor of 3.7 based on the deuterium labeling of alanine during de novo synthesis; and t represents the time between biopsies on *days 4* and *7*.

Statistics. All data are expressed as means ± SE. A one-factor repeated-measures ANOVA with time as within-subjects factor was performed for the analysis of body water and plasma enrichments over time. A two-factor repeated-measures ANOVA (time × treatment) with time and treatment group (exercise vs. control leg) as within-subjects factor was performed for the analysis of intramuscular free [^{2}H]alanine and mixed muscle protein bound [^{2}H]alanine enrichments. Upon identification of a significant time × treatment interaction, Tukey post hoc testing was used to identify differences. FSR values were compared between treatments using paired *t*-tests. Pearson's *r* product moment correlations were performed to examine the linear relationship among precursor pool enrichments, intramuscular free [^{2}H]alanine enrichment between treatments and FSR values calculated with different precursor pools. Statistical significance was set at $P < 0.05$. All calculations were performed using SPSS 21.0 (Chicago, IL).

RESULTS

Physical activity and diet. Subjects' baseline characteristics are presented in Table 1. During the 3-day resistance-type exercise training protocol, subjects consumed on average 11.2 ± 0.4 MJ, with 43 ± 3 energy% (En%) as carbohydrate, 30 ± 2 En% as fat, and 24 ± 3 En% as protein. Dietary protein intake averaged 1.7 ± 0.2 g·kg body wt^{-1}·per day^{-1}. In addition, subjects ingested 30 g whey protein immediately after each exercise bout. During the 3-day resistance-type exercise protocol, subjects averaged 7,266 ± 686 steps/day.

Precursor pools. Body water deuterium enrichment (Fig. 2*A*) reached 0.62 ± 0.02% on *day 3*, which represented the start of the maintenance-dosing period. Body water enrichments remained in steady state over the duration of the experiment ($P > 0.05$) and averaged 0.70 ± 0.03% during the 3-day resistance-type exercise training protocol (*days 4–7*), when muscle protein synthesis rates were assessed. Plasma free [^{2}H]alanine enrichment (Fig. 2*B*) reached 2.35 ± 0.08 mole percent excess (MPE) on *day 3* of the experiment. Plasma free [^{2}H]alanine remained in steady state over the duration of the experiment ($P > 0.05$), and averaged 2.57 ± 0.11 MPE during the assessment of muscle protein synthesis rates. Intracellular free [^{2}H]alanine enrichments (Fig. 3*A*) averaged 1.84 ± 0.06 MPE on *day 4* and increased to 2.15 ± 0.08 MPE on *day 7* (time effect: $P < 0.05$), with no differences detected between legs at each time point (time × treatment: $P > 0.05$).

Endogenous deuterium labeling dynamics. Plasma free [^{2}H]alanine enrichment was 3.74-fold greater when compared with body water deuterium enrichment (Fig. 2*C*). Pearson's *r* product moment correlations were performed among body water deuterium enrichments, plasma free [^{2}H]alanine enrichments, and intramuscular free [^{2}H]alanine enrichments collected at the same time point. A significant positive ($r = 0.778$; $P < 0.0001$) correlation was detected between body water deuterium and plasma free [^{2}H]alanine enrichments (Fig. 2*D*). A significant positive ($r = 0.919$; $P < 0.001$) correlation was detected between body water deuterium and intramuscular free [^{2}H]alanine enrichments (*days 4* and *7*) in the control leg (Fig. 3*B*). A significant positive ($r = 0.845$; $P < 0.001$) correlation was detected between body water deuterium and intramuscular free [^{2}H]alanine enrichments (*day 7*) in the exercised leg (Fig. 3*C*). A significant positive ($r = 0.808$; $P < 0.0001$) correlation was detected between plasma free [^{2}H]alanine and intramuscular free [^{2}H]alanine enrichments (*days 4* and *7*) in the nonexercised, control leg (Fig. 3*D*). A significant positive ($r = 0.820$; $P < 0.001$) correlation was detected between plasma free [^{2}H]alanine and intramuscular free [^{2}H]alanine enrichments (*day 7*) in the exercised leg (Fig. 3*E*). A significant positive ($r = 0.905$; $P < 0.0001$) correlation was detected between intramuscular free [^{2}H]alanine enrichments in the exercised and nonexercised control leg (*day 7*) (Fig. 3*F*).

Muscle protein synthesis rates. Muscle protein-bound [^{2}H]alanine enrichments were measured in muscle samples collected on *days 4* and *7* of the experimental protocol in the exercised and nonexercised control leg. Muscle protein bound [^{2}H]alanine enrichments (Fig. 4) averaged 0.0577 ± 0.0051 MPE in the nonexercised control leg and 0.0585 ± 0.0053 MPE in the exercised leg on *day 4* with no differences between legs ($P > 0.05$). Muscle protein bound [^{2}H]alanine enrichments increased over time in both groups and averaged 0.185 ± 0.013 MPE in the nonexercised control leg and 0.214 ± 0.016 MPE in the exercised leg on day 7 ($P < 0.05$ for differences between legs). Muscle protein FSR (in %/day) was calculated using mean body water deuterium enrichment corrected by a factor of 3.7 as precursor (Fig. 5, *A* and *D*), mean plasma free [^{2}H]alanine enrichments as precursor (Fig. 5, *B* and *E*), and mean intramuscular free [^{2}H]alanine enrichments as precursor (Fig. 5, *C* and *F*). When calculated based on body water deuterium enrichments as a precursor, muscle protein synthesis rates were 24 ± 8% higher in the leg that performed resistance-type exercise (1.984 ± 0.118%/day) when compared with the nonexercised control leg condition (1.642 ± 0.089%/day; $P < 0.05$). When calculated based on plasma free [^{2}H]alanine enrichments as a precursor, muscle protein synthesis rates were higher (24 ± 8%) in the exercised leg (1.975 ± 0.108%/day) when compared with the control leg (1.637 ± 0.085%/day; $P < 0.05$). When calculated based on intramuscular free [^{2}H]alanine enrichments as a precursor, mixed muscle protein synthesis rates were 29 ± 8% higher in the exercise leg (2.609 ± 0.150%/day) when compared with the control leg (2.071 ± 0.108%/day; $P < 0.01$). A Pearson's *r* product moment correlation was performed between FSR calculated with body water deuterium enrichment as precursor and FSR calculated with intramuscular free [^{2}H]alanine enrichment as precursor for both legs (Fig. 6). A significant positive ($r = 0.967$; $P < 0.0001$) correlation was detected.

Cell signaling analyses. The phosphorylation status (ratio of phosphorylated to total protein) of key proteins involved in the initiation of muscle protein synthesis is presented in Fig. 7. No significant differences were detected in muscle tissue samples

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collected 24 h after the last exercise session from the exercised and nonexercised control leg.

DISCUSSION

In the present study, we applied deuterated water to assess the impact of resistance-type exercise on muscle protein synthesis rates over multiple days in free-living conditions. Unilateral resistance-type exercise completed on 3 consecutive days resulted in ~25% higher muscle protein synthesis rates in the exercised leg when compared with the contralateral, nonexercised control leg.

Previous studies have applied intravenous stable isotope-labeled amino acid infusions to assess skeletal muscle protein synthesis rates during the acute postexercise recovery phase (2, 11, 12, 15, 41, 42, 62, 63). However, the postexercise recovery phase extends past the traditional 6–12 h tracer infusion assessment period and may be influenced by diurnal factors such as diet (1), hormonal responses (60), and sleep (25, 26). Currently, few studies have assessed the overall skeletal muscle protein synthetic response to resistance-type exercise performed under free-living conditions over multiple days (4, 8, 17, 61). Here, we applied deuterated water to assess the muscle protein synthetic response to resistance-type exercise performed daily over a 3-day assessment period. Our deuterated water-dosing protocol increased body water deuterium enrichment to 0.62 ± 0.02% and [^{2}H]alanine enrichment to 2.35 ± 0.08 MPE after the first dosing day (*day 3*). The body water and plasma enrichments remained in steady state for the remainder of the 3-day assessment period (Fig. 2, *A* and *B*). The labeling coefficient between body water deuterium and plasma free [^{2}H]alanine enrichment was 3.74 ± 0.05 (Fig. 2*C*). This labeling coefficient is directly in line with previous work (20, 43, 61) and reflects the rapid and tightly controlled equilibrium between body water deuterium and the free alanine precursor pool(s). As such, our dosing protocol resulted in deuterium precursor conditions that allowed us to quantify fractional muscle protein synthesis rates by measuring the increase in [^{2}H]alanine incorporation over the 3-day period in the exercised and nonexercised leg muscle tissue.

In the present study, we applied a unilateral leg exercise design and took muscle biopsies to allow direct assessment of the surplus effect of (local) resistance-type exercise on muscle protein synthesis rates under free-living conditions. We found that muscle protein synthesis rates averaged 1.642 ± 0.089%/day (or 0.068 ± 0.004%/h; Fig. 5*D*) in the nonexercised, control leg and 1.984 ± 0.118%/day (0.083 ± 0.005%/h; Fig. 5*D*) in the resistance-type exercised leg. These daily muscle protein synthesis rates agree well with the more acute stable isotope infusion studies, as basal, postabsorptive muscle protein synthesis rates typically range between 0.02 and 0.04%/h (12, 13, 23, 37) and acute postexercise muscle protein synthesis rates have been shown to range between 0.06 and 0.10%/h (12, 16, 29, 41, 45, 46, 62, 63). Despite the relatively high habitual

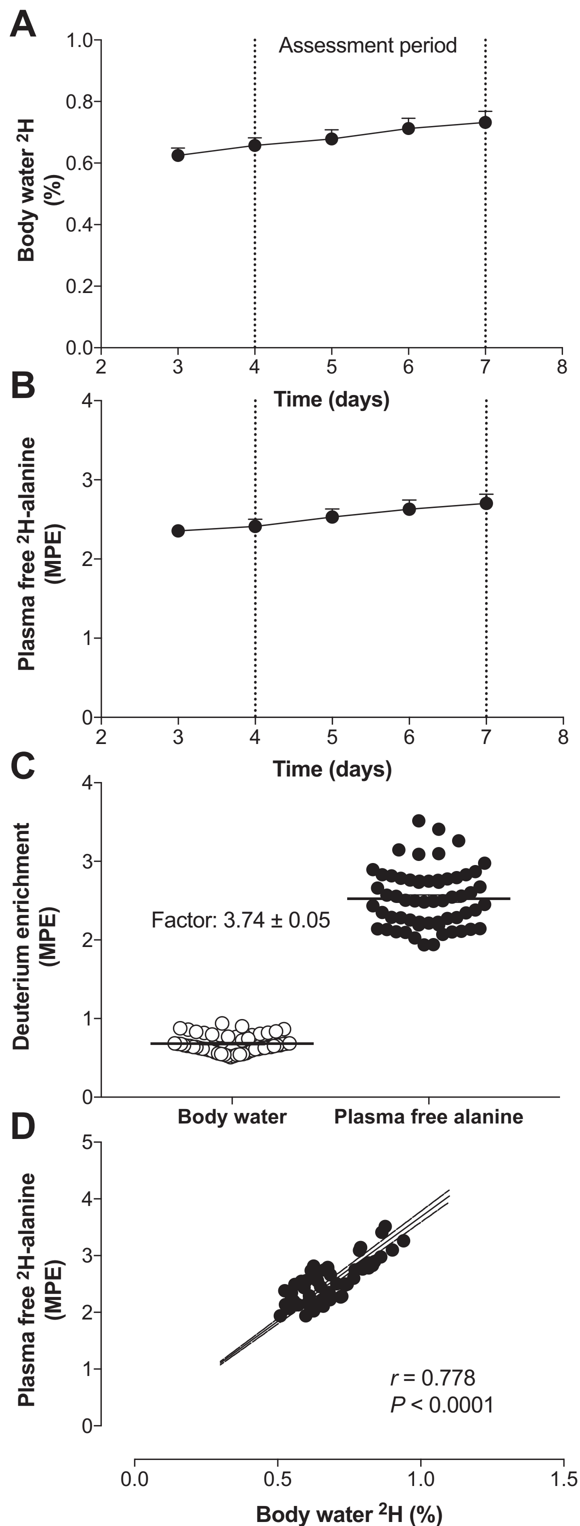

Fig. 2. Body water deuterium enrichment (*A*; %) and plasma free [^{2}H]alanine enrichment [*B*; mole percent excess (MPE)] displayed over time during oral deuterated water dosing in young men (n = 12). Dosing began on *day 2*, subjects were in steady state between *days 3* and *7*. Values represent means ± SE. Data were analyzed using a one-way repeated-measures ANOVA. Body water deuterium and plasma free [^{2}H]alanine enrichment in corresponding samples expressed in relation to one another (*C*; %body water deuterium, MPE for plasma free [^{2}H]alanine). Correlation between body water deuterium enrichment and plasma free [^{2}H]alanine enrichment of corresponding samples (*D*; %body water deuterium, MPE for plasma free [^{2}H]alanine). The solid line indicates the linear regression line of best fit, the dashed lines represent the 95% confidence interval. A significant positive correlation was observed (P < 0.0001).

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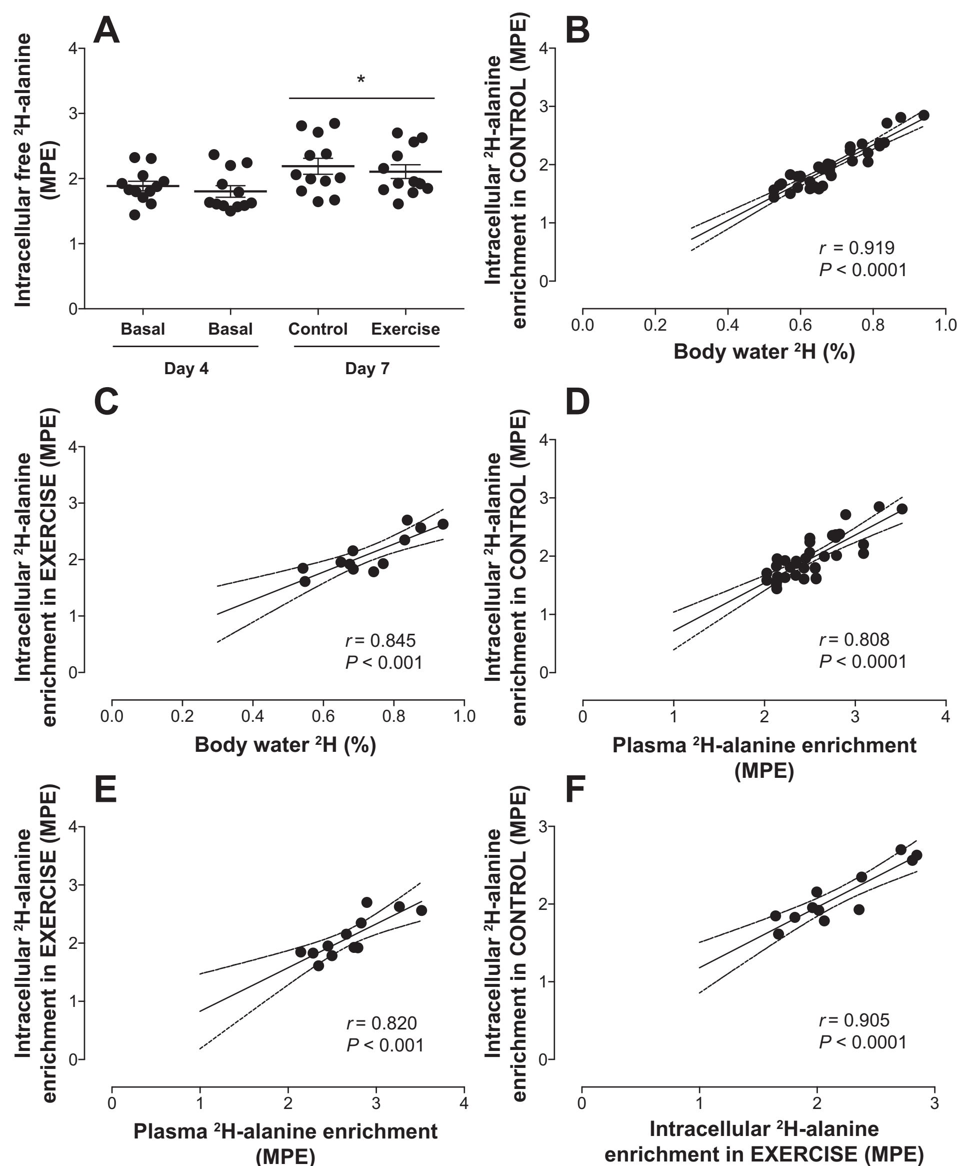

Fig. 3. *A*: intramuscular free [^{2}H]alanine enrichment (MPE) from the vastus lateralis muscle of each leg at *day 4* (basal) and *day 7* (control and exercise). Data were analyzed using a two-way repeated-measures ANOVA with time and treatment leg as within subject factors. *Significantly different compared with basal ($P < 0.01$). Multiple correlation analyses were conducted between precursor pools. *B*: intramuscular free [^{2}H]alanine enrichment in control leg (MPE) was positively correlated with body water deuterium enrichment (%; $P < 0.001$). *C*: intramuscular free [^{2}H]alanine enrichment in exercise leg (MPE) was positively correlated with body water deuterium enrichment (%; $P < 0.001$). *D*: intramuscular free [^{2}H]alanine enrichment in control leg (MPE) was positively correlated with plasma free [^{2}H]alanine enrichment (MPE; $P < 0.001$). *E*: Intramuscular free [^{2}H]alanine enrichment in exercise leg (MPE) was positively correlated with plasma free [^{2}H]alanine enrichment (MPE; $P < 0.001$). *F*: intramuscular free [^{2}H]alanine enrichment in control leg (MPE) was positively correlated with intramuscular free [^{2}H]alanine enrichment in exercise leg (MPE; $P < 0.001$). The solid line indicates the linear regression line of best fit, and the dashed lines represent the 95% confidence interval.

physical activity level and high protein intake of our young male subjects, our data demonstrate 24 ± 8% higher muscle protein synthesis rates in the leg that performed 45 min of resistance-type exercise daily when compared with the nonexercised, control leg ($P < 0.05$). These findings align well with recent work from Wilkinson et al. (61), who applied a deuterated water approach to show that unilateral resistance-type exercise results in 33% higher muscle protein synthesis rates over a 4-day period when compared with the contralateral, nonexercised control leg. We extend on their findings by showing 24% higher muscle protein synthesis rates in the exercised compared with the control leg despite the application of a different deuterated water-dosing strategy, a different exercise protocol, and measurement mixed muscle as opposed to myofibrillar protein synthesis rates.

Recent work by Mitchell et al. (36) has demonstrated that muscle protein synthesis rates assessed during acute postexercise recovery are not predictive of absolute changes in muscle mass observed during more prolonged resistance type exercise training. This finding has garnered attention in the field as the interpretation of acute measures of postexercise muscle protein synthesis rates may be difficult to align with long-term changes in skeletal muscle mass (3, 33, 34). However, it is not surprising that assessment of muscle protein synthesis in the acute postexercise recovery phase under controlled laboratory settings are not predictive of long-term changes in muscle mass. For instance, it has been well described that resistance-type exercise increases postprandial muscle protein synthesis rates by as much as 50–100% during the early stages of postexercise recovery (12, 41, 42, 63). Our observation of 24% higher muscle protein synthesis rates in the exercised vs. control leg when assessed over an extended 3-day period reflects the impact of other anabolic factors on the regulation of muscle protein synthesis rates throughout the day(s), which likely include dietary protein intake, habitual physical activity, sleep, hormonal fluctuations, and/or transient anabolic signaling responses. As all these factors contribute to muscle mass regulation, it is obvious that the application of the deuterated water

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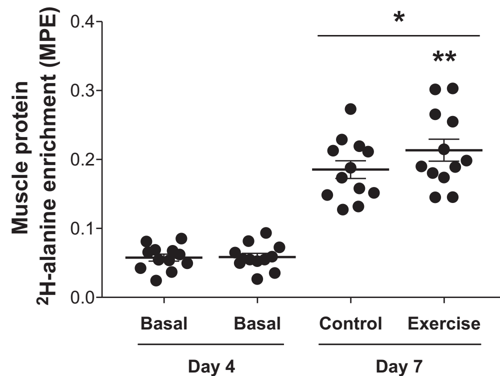

Fig. 4. Muscle protein bound [^{2}H]alanine enrichment (MPE) from the vastus lateralis muscle of each leg at *day 4* (basal) and *day 7* (control and exercise). The control leg completed normal activities of daily living and the exercise leg completed unilateral resistance-type exercise (leg press and leg extension) once per day for 3 days. Data were analyzed using a two-way repeated measures ANOVA with time and treatment leg as within subject factors. *Significantly different compared with basal ($P < 0.05$). **Significantly different compared with control ($P < 0.05$).

method likely provides a more accurate tool to assess the impact of (more prolonged) exercise interventions on muscle protein synthesis and the associated changes in muscle mass (17). However, future work is still warranted to confirm whether muscle protein synthesis rates assessed over multiple days are more predictive of subsequent net changes in muscle mass during various long-term interventions.

Resistance-type exercise activates intramuscular signaling proteins that regulate protein translation-initiation, with mTOR and its downstream targets, S6 kinase, RS6, and 4E-BP1, being of particular relevance. Here, we observed no differences in the phosphorylation status of these proteins between the exercised and nonexercised control leg 24 h following the last exercise session. Although our biopsy timing does not allow us to assess transient changes in the phosphorylation status of these proteins during recovery from exercise, our findings support an increasing number of studies that demonstrate the inability to align the phosphorylation status of key translation initiation factors with the physiological outcome of muscle protein synthesis rates (14, 24, 59, 63).

The choice of precursor pool is an important consideration when assessing muscle protein synthesis rates (10, 52). Although it would be ideal to use the true precursor (aminoacyl-tRNA enrichment) for assessing muscle protein synthesis rates, accurate analysis is technically difficult to perform and would require tissue sample sizes too large to collect (>1,000 mg) (58, 64). As such, researchers applying stable isotope amino acid infusion protocols generally use the plasma or intramuscular free amino acid pools as a surrogate for aminoacyl-tRNA enrichment (52). Prior work applying the deuterated water approach has suggested that body water deuterium enrichment, assessed in saliva or urine samples, can be used as a reliable surrogate precursor for calculating muscle protein synthesis rates (31, 43, 61). However, few data exist on the reliability of using body water deuterium enrichment as a precursor as opposed to using intramuscular free [^{2}H]alanine enrichment. In the present study, we calculated fractional synthetic rates based on three different precursor pools {i.e., body water deuterium (Fig. 5*D*), plasma free [^{2}H]alanine (Fig. 5*E*), and intramuscular free [^{2}H]alanine (Fig. 5*F*) enrichments}. Across all calculations we found that resistance-type exercise resulted in 24–29% higher muscle protein synthesis rates when compared with the nonexercised control leg, with no differences detected between the choice of precursor ($P > 0.05$). Furthermore, we found a strong positive correlation ($r = 0.967$; $P < 0.0001$)

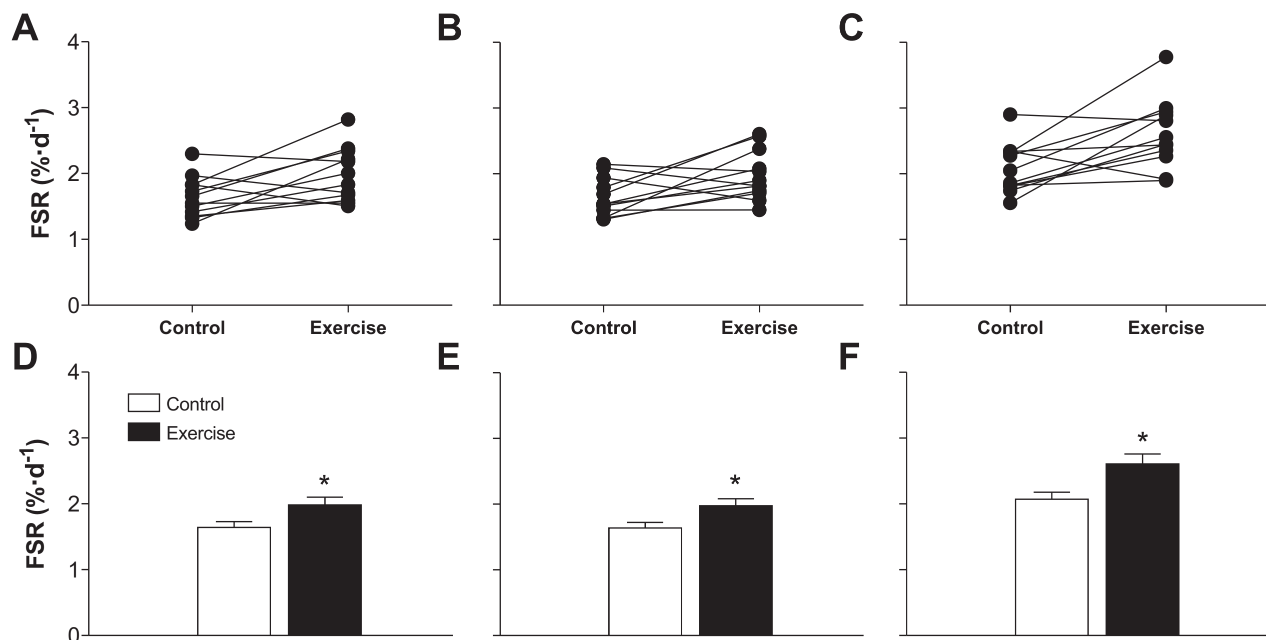

Fig. 5. Mixed muscle protein fractional synthetic rates (FSR in %/h) assessed in young men while performing bouts of unilateral resistance-type exercise on 3 consecutive days using deuterated water dosing. Fractional synthetic rates were calculated using body water deuterium (*A* and *D*), plasma free [^{2}H]alanine (*B* and *E*), and intramuscular free [^{2}H]alanine enrichment (*C* and *F*) as precursor. Individual values represented in *A–C*. Values in *D–F* are represented as means ± SE. Data were analyzed with Student's unpaired t-test. *Significantly different compared with control leg ($P < 0.05$).

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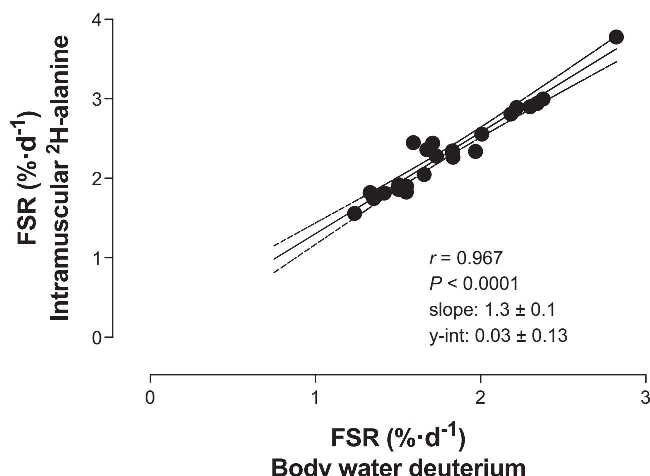

Fig. 6. Pearson's r product moment correlation performed between mixed muscle protein fractional synthetic rates (FSR in %/h) calculated with body water deuterium enrichment as precursor and intramuscular free [^{2}H]alanine enrichment as precursor. The solid line indicates the linear regression line of best fit, and the dashed lines represent the 95% confidence interval. A significant positive correlation was detected.

between muscle protein synthesis rates calculated using body water deuterium as a precursor and muscle protein synthesis rates calculated using intramuscular free [^{2}H]alanine as a precursor (Fig. 6). These findings reinforce the prior suggestions that body water deuterium enrichment represents an appropriate precursor for calculating muscle protein fractional synthetic rates (31, 43, 61). Saliva sampling provides a substantial advantage over repeated blood and/or muscle tissue sampling and reduces the burden on test subjects, adding to the benefits of using the deuterated water approach to study muscle protein synthesis rates in more prolonged, practical, and real-life (exercise) settings.

The findings presented in this study further support the use of deuterated water dosing as a practical approach to assess changes in muscle protein synthesis rates during interventions performed over a period of several days to even weeks (7, 8, 21, 30, 38, 48, 49, 61). We consider the single-dosing day and maintenance-dosing period as an effective means to rapidly increase deuterium enrichment and maintain precursor steady state for days to weeks of an assessment period. Furthermore, the observation that body water deuterium rapidly equilibrates with free [^{2}H]alanine precursor pools permits the reliable assessment of muscle protein synthesis rates by collecting muscle biopsies and saliva samples. As such, the deuterated water method provides a practical approach to study test participants in a nonlaboratory, free-living setting. This approach will serve to provide novel insight into current gaps in our understanding into the dynamic nature of skeletal muscle reconditioning under different conditions.

In conclusion, daily resistance-type exercise stimulates local muscle protein synthesis rates by ~25% when assessed over multiple days in vivo in humans. The application of deuterated water provides a practical approach to assess the muscle protein synthetic response to various interventional strategies performed over multiple days to weeks.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

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

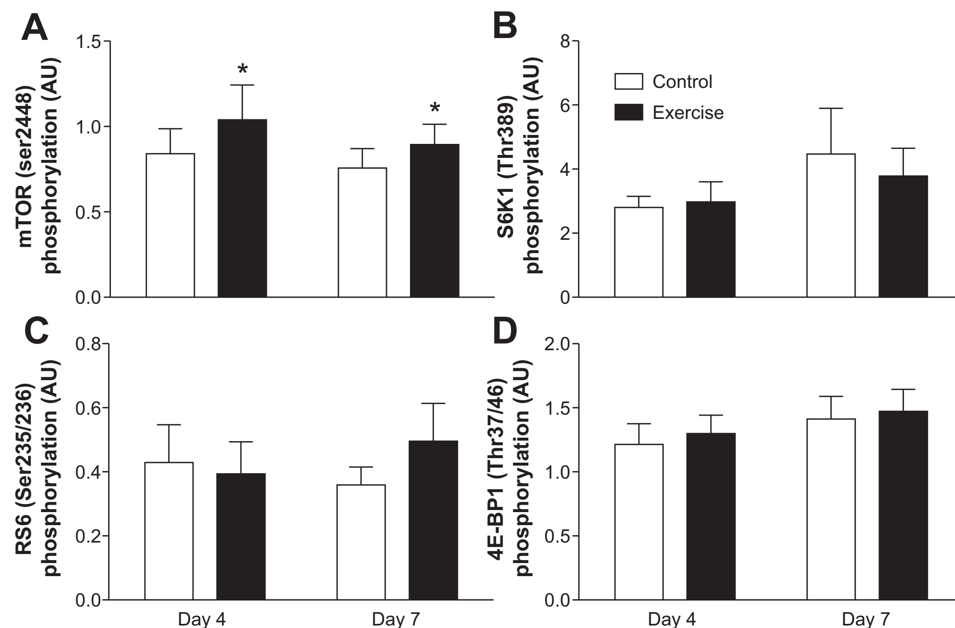

Fig. 7. Muscle phosphorylation status (ratio of phosphorylated to total protein) of mammalian target of rapamycin (mTOR; *A*), S6 protein kinase 1 (S6K; *B*), ribosomal protein S6 (RS6; *C*), and eukaryotic translation initiation factor 4E-binding protein-1 (4E-BP1; *D*) in young men (n = 12) immediately before (*day 4*) and 24 h after completing the last unilateral exercise bout from the exercised and nonexercise control leg (*day 7*). Data were analyzed with a two-way repeated-measures (time × treatment leg) ANOVA. *A*: time effect: $P > 0.05$, treatment leg effect: $P < 0.05$, time × treatment group: $P > 0.05$. *B*: time effect: $P > 0.05$, treatment effect: $P > 0.05$, time × treatment group: $P > 0.05$. *C*: time effect: $P > 0.05$, treatment effect: $P > 0.05$, time × treatment group: $P > 0.05$. *D*: time effect: $P > 0.01$, treatment effect: $P > 0.01$, time × treatment group: $P > 0.05$ *Significantly different compared with control leg ($P < 0.05$).

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