The impact of disuse and high-fat overfeeding on forearm muscle amino acid metabolism in humans

Benjamin T Wall[1], Ana M Cruz[1,2], Britt Otten[1], Mandy V Dunlop[1], Jonathan Fulford[2], Craig Porter[3], Doaa Reda Abdelrahman[3], Stephens B Francis[1] and Marlou L Dirks[1]

[1]*Department of Sport and Health Sciences, College of Life and Environmental Sciences, University of Exeter, UK*

[2]*Exeter Medical School, University of Exeter, UK*

[3]*Department of Surgery, University of Texas Medical Branch & Metabolism Unit, Shriners Hospital for Children, Galveston, TX 77550-1220, USA*

Disclosure summary: none of the authors have any conflicts of interest to disclose.

Registered on clinicaltrials.gov as NCT02980952

Funding sources: The Physiological Society

Corresponding author:

Marlou L Dirks, PhD

Department of Sport and Health Sciences

College of Life and Environmental Sciences

St Luke's Campus, Heavitree Road

University of Exeter

Exeter, EX1 2LU

UK

Tel: +44 (0)139 272 5496

Email: m.dirks@exeter.ac.uk

Abstract

Context: Anabolic resistance is mechanistically implicated in muscle disuse atrophy. **Objective:** Assess whether anabolic resistance is associated with reduced postprandial amino acid uptake or exacerbated by excess lipid availability. **Design, setting, participants and interventions**: Twenty men underwent 7 days of forearm immobilization while consuming a eucaloric (CON; *n*=11) or high-fat overfeeding (HFD; *n*=9; 50% excess energy as fat) diet (parallel design) within our Nutritional Physiology Research Unit. **Main outcome measures**: Pre- and post- immobilization we measured forearm muscle cross sectional area (aCSA), and postabsorptive and postprandial (3 h post ingestion of a liquid, protein rich, mixed meal) forearm amino acid metabolism using the arterialised venous-deep venous balance method and infusions of L-[*ring*-$^{2}H_{5}$]phenylalanine and L-[1-^{13}C]leucine. **Results:** Immobilization did not affect forearm muscle aCSA in either group, but tended to reduce postabsorptive phenylalanine (*P*=0.07) and leucine (*P*=0.05) net balances equivalently in CON and HFD. Mixed meal ingestion switched phenylalanine and leucine net balances from negative to positive (*P*<0.05), an effect blunted by immobilization (*P*<0.05) and to a greater extent in HFD than CON (*P*<0.05). Pre-immobilization, meal ingestion increased leucine rates of disappearance (R_d; *P*<0.05), with values peaking at 191% (from 87±38 to 254±60 µmol·min^{-1}·100 mL forearm volume^{-1}) and 183% (from 141±24 to 339±51 µmol·min^{-1}·100 mL^{-1}) above postabsorptive rates in CON and HFD, respectively, with meal induced increases not evident post-immobilization in either group (*P*>0.05). **Conclusions:** Disuse impairs the ability of a protein-rich meal to promote positive muscle amino acid balance, which is aggravated by dietary lipid oversupply. Moreover, disuse reduced postprandial forearm amino acid uptake; however, this is not worsened under high-fat conditions.

Keywords: Disuse, arterio-venous balance, skeletal muscle, amino acid uptake, stable isotopes, anabolic resistance, atrophy

Introduction

Skeletal muscle mass is largely regulated by physical activity. Withdrawal of muscle contraction during disuse results in rapid muscle atrophy within one week (1). Negative health consequences associated with muscle disuse atrophy (e.g. insulin resistance and reduced function) are well documented (2, 3) and make a detailed understanding of the underlying mechanisms a key area of investigation.

Disuse induced declines in postabsorptive muscle protein synthesis (MPS) rates and the blunted MPS response to dietary protein ingestion (termed 'anabolic resistance') are widely viewed as key mechanisms responsible for muscle disuse atrophy (4-6). While anabolic resistance could originate within myocellular anabolic signalling pathways (4, 7), it may also be a consequence of reduced systemic or local amino acid delivery. However, whether disuse is associated with impaired muscle amino acid uptake has not been established.

Muscle amino acid uptake is contingent on several transporter proteins that generally reside on the cell membrane (8). Dietary protein ingestion leads to a rapid increase in gene and protein expression of these transporters, and this is often assumed to reflect intracellular amino acid flux and the subsequent stimulation of MPS rates (8-10). The rise in amino acid transporter expression following protein ingestion has been shown to be blunted with disuse in some (6) but not all (4) studies. This discrepancy likely highlights the difficulties in extrapolating static molecular measurements to a dynamic physiological process.

We have previously discussed that anabolic resistance could result from disuse *per se*, or indirectly as a consequence of (or exacerbated by) altered nutritional status (11). For instance, during disuse there is an immediate reduction in (local) energy expenditure accompanied by excess lipid delivery to and/or uptake by inactive tissue (12). A (negative) regulatory role of lipid availability upon anabolic sensitivity is also implied by reports of anabolic resistance being present in overweight and

obese individuals (10, 13-15), humans subjected to intralipid infusions (16) and rodents overfed a high-fat diet (17, 18). However, these are not uniform findings; some studies report no differences in anabolic sensitivity between lean and obese older adults (19), no impact (20) or even a potentiating effect (21) of acute fat co-ingestion with protein on postprandial MPS rates, and recent data reveal neither acute nor short-term dietary fat overload impaired postprandial MPS rates in older adults (22). While such a link between lipid availability and anabolic sensitivity may be mechanistically explained by the intracellular deposition of lipid (metabolites) blunting anabolic signalling pathways or impaired cellular delivery of amino acids (4, 16, 18), the inconsistency of findings requires further investigation.

In the present study, therefore, we investigated how immobilization modulated the ability of a protein-rich meal to promote a positive net amino acid balance and stimulate uptake of two essential amino acids across the forearm tissue bed by applying an arterialised venous-deep venous (AV-V) balance approach combined with the dual intravenous infusions of L-[*ring*-$^{2}H_{5}$]phenylalanine and L-[1-^{13}C]leucine under eucaloric and (high-fat) hypercaloric conditions. We hypothesised that disuse would impair forearm amino acid uptake and this would be associated with a reduced ability of a protein-rich meal to promote a positive net amino acid balance, effects that would be exacerbated by a high-fat diet.

Methods

Participants

Twenty-two healthy, young men were included in the present study, though due to technical issues during data collection our final dataset comprises twenty individuals. Before inclusion into the study, participants completed a routine medical screening visit to ensure suitability for acceptance onto the study. Participants fulfilling one or more of the following criteria were excluded: age below 18 or over 40 y, BMI below 18.5 or over 30 $kg \cdot m^{-2}$, metabolic impairment (e.g. type 1 or 2 diabetes), hypertension, cardiovascular disease, chronic use of any prescribed over the counter pharmaceuticals, regular use of nutritional supplements, metallic implants, a personal or family history of thrombosis, any previous motor disorders, any disorders in lipid metabolism, presence of an ulcer in the stomach or gut, and severe kidney problems. All participants were fully informed on the nature and risks of the experiment before written informed consent was obtained. During screening height and weight were measured, and body composition was determined by Air Displacement Plethysmography (Bodpod; Life Measurement, Inc., Concord, CA, USA). Volunteers also completed the International Physical Activity Questionnaire, IPAQ (23). The present study was part of a larger project investigating the impact of forearm immobilization and high-fat overfeeding on muscle substrate metabolism, registered on clinicaltrials.gov as NCT02980952. Some data from this work has previously been published (12). The study was approved by the Department of Sport and Health Sciences, University of Exeter's Ethical Committee (reference number 161026/B/09) in accordance with the Declaration of Helsinki (version October 2013).

Experimental overview

Participants undertook two metabolic test days separated by a one week period of dietary control and forearm immobilization. Participants visited the laboratory for a baseline metabolic test day (pre immobilization) during which muscle anatomical cross-sectional area (aCSA) of the forearm was assessed using MRI and postabsorptive and postprandial amino acid kinetics across the forearm tissue bed were assessed using the combination of intravenous stable isotope labelled amino acid infusions and the arterialised venous-deep venous (AV-V) forearm balance method (12, 24). Minimally 3 and maximally 32 days later (mean; 10±2 days), participants attended the laboratory for the application of a forearm cast to bring about disuse of the forearm muscle tissue bed. This visit signified the beginning of a 7-day immobilization period. During these 7 days, participants were provided with a fully controlled eucaloric (CON; *n*=11) or a high-fat overfeeding diet providing 50% excess energy from fat (HFD; *n*=9) (details below). Following 7 days of immobilization and dietary intervention, an identical metabolic test day was performed to assess the effects of disuse and diet on forearm muscle aCSA and amino acid metabolism. The forearm cast was removed following the final test day signifying the end of the experiment.

MRI scanning and assessment of forearm muscle mass

Immediately following cast application, and on the morning after 7 days of immobilization, participants underwent an MRI scan of both arms to determine forearm muscle volume and aCSA using a 1.5T Philips Intera system. For this, participants lay on the scanning table in a prone, face-down position and with both arms extended overhead. Both arms were scanned separately using a Turbo Spin Echo (TSE) sequence (in-plane resolution 1x1 mm, Echo time=15 ms, Repetition time= 421 ms, 3 signal averages) with sixty 5 mm slices, with 1 mm gaps, acquired of each arm perpendicular to its long axis.

Forearm muscle aCSA at 2 cm below the bony landmark of the elbow was determined as a measure of forearm muscle mass from both arms and for each visit via a semi-automatic thresholding technique within the Philips scanner toolbox that selectively highlighted the muscle tissue. For the adjustment of forearm volume within our AV-V modelling formulae, muscle volume was calculated by firstly determining the muscle aCSA in all slices that contained forearm muscle slices 1 to *n* (i.e. muscle areas $aCSA_1$ to $aCSA_n$) by the same technique as for the single slice. Then, to take account of slice thickness and slice gaps, volume (in cm^3) can be determined via the following equation:

$$Muscle\ volume =$$

$$aCSA_1 \times 0.55 + aCSA_n \times 0.55 + 0.6 \times (aCSA_2 + aCSA_3 + aCSA_3 + \cdots etc \ldots + aCSA_{n-1})$$

Experimental test day

Participants arrived at the laboratory at 8:00 h in an overnight fasted state for the two experimental test days. Following MRI scanning, body weight was measured with a digital balance with an accuracy of 0.05 kg (Seca, Hamburg, Germany). Participants rested on the bed in a semi-supine position for the entire experimental test day. First, a cannula was placed retrogradely into a dorsal hand vein of the non-immobilized (selected as the dominant) hand, and the hand was then placed in a heated hand warmer (55°C) for the remainder of the test day to repeatedly sample arterialised venous blood (25). A second cannula was placed retrogradely into a deep-lying antecubital vein of the arm previously identified to be immobilised (i.e. non-dominant arm) to sample venous blood drainage from the forearm muscle bed (12, 24, 26, 27). Doppler ultrasound was used to locate the anatomy of this vein to assist in the cannulation. Both these cannulas were kept patent by a continuous saline drip. To confirm the sampling lines accurately reflected arterialised and venous blood we determined oxygen saturation levels in both lines prior to beginning a test day using a benchtop online blood gas analyser (HOX Ultra blood gas analyser, NOVA Biomedical, Waltham, MA,

USA). Oxygen saturations were 93±1 and 67±5% (P<0.05; pre-immobilization) and 92±1 and 63±4% (P<0.05; post-immobilization) in arterialised venous and venous blood, respectively, with no effect of immobilization (or interaction) on either parameter (both P>0.05). A third cannula was then inserted anterogradely into an antecubital vein of the non-immobilised arm in preparation for stable isotope infusion. Following a baseline venous blood collection to establish background tracer enrichments, the plasma phenylalanine and leucine pools were primed with a single intravenous stable isotope bolus (0.5 mg·kg^{-1} L-[*ring*-$^{2}H_5$]phenylalanine, 1.0 mg·kg^{-1} L-[1-^{13}C]leucine) after which a continuous stable isotope infusion was commenced (0.5 mg·kg^{-1}·h^{-1} L-[*ring*-$^{2}H_5$]phenylalanine, 1.0 mg·kg^{-1}·h^{-1} L-[1-^{13}C]leucine) for the duration of the test day. After 100 and 120 min of the stable isotope infusion, parallel AV-V samples were collected at exactly the same time by two investigators to assess forearm metabolism in the postabsorptive state. At t=0 min, i.e. 120 min after the start of the stable isotope infusion, participants ingested an Ensure Plus drink (Abbott Nutrition, Lake Forest, IL, USA) containing 1.0 g carbohydrates, 0.3 g protein, and 0.2 g fat per kg body weight (mean 21.8±0.8 g protein). Further AV-V blood was sampled simultaneously every 20 min during a 3 h postprandial period. Prior to every blood sample, brachial artery blood flow of the (to be) immobilized arm was determined by ultrasound imaging (~12 MHz, Apogee, 1000. SIUI, China). Luminal diameter was imaged 5 cm proximal to the antecubital fossa for a 2 second period. Mean blood velocity was determined at the same anatomic location by integration of the pulsed-wave Doppler signal for a minimum of 8 cardiac cycles (28). Files were analysed semi-automatically using Brachial Analyzer for Research, version 6.8.5 (Medical Imaging Applications LLC, Coralville, IA, USA). Following the 3 h postprandial period, infusions were stopped, cannulas removed and volunteers were asked to stand up from the bed to perform maximal handgrip exercise to determine maximal forearm muscle strength. Briefly, three consecutive measures of maximal handgrip strength of both hands were taken using a hydraulic hand dynamometer (Takei Scientific Instruments. Ltd., Yashiroda, Japan) with subjects standing upright and their arm in a vertical position without touching the side of the body. The highest value achieved was taken as their maximal strength.

Forearm casting

On the morning of the start of the 7 d immobilization period, participants arrived at the laboratory at 7:30 h to have a forearm cast fitted on their non-dominant arm. Firstly, stockinette and undercast padding were applied to protect the skin. Next, a fiberglass cast (Benecast™, BeneCare Medical, Manchester, UK) was fitted to the arm to immobilise the wrist. This resulted in a cast which extended from 5 cm distal of the antecubital fossa to 2 cm proximal of the finger tips. Participants were provided with a sling and instructed to wear that during waking hours to keep the hand elevated above the elbow. A waterproof cover was provided to keep the cast dry whilst showering.

Dietary control

Prior to the immobilization period participants were instructed to keep a food diary for three consecutive days, including two weekdays and one weekend day. Habitual energy and macronutrient intake was calculated from these food diaries using online licensed software (29). During the seven days of forearm immobilization participants received a fully controlled diet from the research team. All meals and snacks were provided whereas water and non-caloric drinks were allowed *ad libitum*. Energy requirements were calculated as basal metabolic rate (estimated via the Henry equations; 30)) multiplied by an activity factor calculated from the previously completed IPAQ (23). Participants in the CON group received an individually tailored energy-balanced diet clamped at 1.2 g protein·kg^{-1} body weight·d^{-1}. Once weight-adjusted protein intake was clamped, the target macronutrient composition was 50-55 of total daily energy intake (en%) from carbohydrate, 30-35 en% from fat, 10-15 en% from protein, and 2 en% from dietary fibre. Participants in the HFD group received a high-fat diet providing 50% excess energy from fat, with absolute protein intake matched to the CON group at 1.2 g protein·kg^{-1} body weight·d^{-1}. Consequently, target macronutrient

composition was 34-36 en% carbohydrate, 54-56 en% fat, 7-9 en% protein, and 1-2 en% dietary fibre. Compliance with the nutritional intervention was assessed via completed 7 day food diaries, returned food containers and daily contact (either in person or by phone) by a member of the research team.

Sample collection and analyses

Arterialised venous and deep-venous blood samples were collected for determination of plasma phenylalanine and leucine concentrations, plasma L-[*ring*-$^{2}H_5$]phenylalanine and L-[1-^{13}C]leucine enrichments and insulin concentrations. To do this, an aliquot (~4 mL) of each sample was collected into chilled BD Vacutainer® PST Lithium Heparin tubes and immediately centrifuged at 2,900*g* at 4°C for 10 min to obtain blood plasma that was immediately frozen in liquid nitrogen. A second aliquot (~2 mL) of each blood sample was collected into BD Vacutainer® SST II tubes and left to clot at room temperature for ≥30 min and then centrifuged at 2,900*g* at 4°C for 10 min to obtain blood serum samples that was snap frozen in liquid nitrogen. Serum insulin concentrations were analysed using a commercially available kit (DRG Insulin ELISA, EIA-2935, DRG International Inc, Springfield, USA) and these data have been published, in part, previously (12). Plasma leucine and phenylalanine concentrations, and plasma L-[ring-$^{2}H_5$]-phenylalanine [1-^{13}C]leucine enrichments were determined by gas chromatography-mass spectrometry (GC-MS) as described previously (31). Briefly, 10 µL internal standards of $^{13}C_9{}^{15}N$-phenylalanine and $^{13}C_6{}^{15}N$-leucine were added to the samples. The plasma was deproteinised on ice with 500 µL of 15% 5-sulfosalicylic acid. Free amino acids were purified using acid-washed cation exchange columns (AG 50W-X8 resin; Bio-Rad, CA, USA), with the amino acids being eluted from the column with 8 mL of 2N ammonium hydroxide. The eluate was then dried under vacuum with a Speed-Vac rotary dryer (Savant Instruments, Farmingdale, NY, USA). In order to derivatize the plasma sample, 40 µl MTBSTFA + 1% tert-butyl-dimethylchlorosilane and 40 µl acetonitrile were added to the dry samples, vortexed and heated at 95 °C for 40 min (32). The

samples were analyzed by GC-MS (7890 GC coupled with a 5975 inert MSD; Agilent Technologies, Santa Clara, CA, USA) in duplicates using electron impact ionization and selected ion monitoring for measurement of isotope ratios (31). One microliter of the sample was injected in splitless mode (injector temp. 280°C). Peaks were resolved using an HP5-MS 30m × 0.25mm ID × 0.25µm capillary column (Agilent). Helium was used as carrier gas at 1.2 mL/min constant flow rate. The temperature ramp was set from 80 – 245 °C at 11°C/min, then to 280 °C at 40 °C/min (31). Selected ion recording conditions were used to monitor fragments m/z 336, with 341 and 346 for phenylalanine and m/z 302, 303 and 309 for leucine.

Arterialised venous-deep venous methodology and calculations

We applied an AV-V model to assess forearm amino acid metabolism in the present study. We (12, 24) and others (33, 34) have used this approach previously to assess dynamic carnitine, glucose, fatty acid and/or amino acid metabolism *in vivo* in humans. Two pool arterialised venous-deep venous amino acid kinetics across the forearm tissue bed were calculated according to the principles and calculations detailed in Wolfe & Chinkes (35), used previously in the literature (36, 37) and discussed briefly below.

Arterial amino acid concentrations do not differ substantially across the body (i.e. in different arteries). Moreover, arterialised venous blood has been shown as an accurate reflection of brachial arterial concentrations for dynamic amino acid kinetics (35, 38, 39). We were measuring blood flow non-invasively which therefore provided no impediment to expected arterial amino acid concentrations. Moreover, we chose to use arterialised venous blood as a less invasive means to represent arterial delivery of substrates to the forearm tissue bed. Implicit in this approach to assessing *muscle* metabolism is the assumption that our sampling sites represent delivery and drainage primarily from muscle tissue and not superficial (e.g. fat, skin) tissues. Previous data

indicate that at least 85% of arterial delivery is to the muscle tissue (34, 40). Though this could be expected to introduce a slight (but systematic) overestimation of *muscle* metabolic balance, it is also true that the deep venous effluent likely represents a similar contribution from non-muscle tissues. While acknowledging these assumptions we subsequently refer to the metabolism measured across the forearm as *muscle* metabolism.

Forearm net balances (NB) were calculated for both phenylalanine and leucine according to the Fick principle (*equation 1*). Specifically, the net balance of a substrate (i.e. in this case phenylalanine or leucine) is equal to the rate that it enters the tissue minus the rate that it exits the tissue. In turn, the rate of entry is determined by the arterial concentration multiplied by the blood flow, whereas the rate of exit is equivalent to venous concentration multiplied by the blood flow. The values were then adjusted for a participants' forearm volume such that data are expressed as $\mu mol \cdot min^{-1} \cdot 100$ mL forearm $volume^{-1}$. Therefore:

$$(1) \quad NB = BF \times (C_A - C_V)$$

(where NB is the net balance, BF is blood flow, C_A is the arterialised venous concentration and C_V is the deep venous concentration of the specific amino acid adjusted for non-steady state conditions (41, 42)).

We selected phenylalanine since this essential amino acid is not metabolised or synthesised in skeletal muscle and therefore this gives us an accurate reflection of the 'anabolic state' of the muscle tissue bed (i.e. 'net' MPS; MPS-MPB). Since leucine is preferentially oxidised within muscle, determining leucine balance is less reflective of anabolic state *per se*. However, we also determined leucine kinetics to gain additional insight into a key nutritional anabolic signalling amino acid (43). To measure muscle phenylalanine and leucine uptakes, and muscle protein breakdown rates, we applied primed continuous intravenous infusions of L-

[*ring*-$^{2}H_5$]phenylalanine and [1-^{13}C]leucine. This allows the calculation of amino acid rates of disappearance (R_d; *equation 2*) and rates of appearance (R_a; *equation 3*) into and from the forearm, respectively. In this model, therefore, R_d reflects irreversible amino acid uptake from the plasma. While extrapolation of R_d is often used as a proxy for MPS (especially for phenylalanine where oxidation can be ignored), the lack of tissue sampling in the present approach led to a consequent lack of information of tracer enrichment within the intracellular space and/or the muscle protein bound fraction. Particularly with disuse, there is reason to believe that anabolic resistance may be characterised by a reduced ability of muscle tissue to incorporate free intracellular amino acids (i.e. already taken up into the muscle cell) into polypeptide chains on the ribosome and thus to exist as functional bound muscle protein (5, 44). As a result, in the present work we consider R_d equivalent to amino acid uptake *only* and do not presume to be able to directly quantify MPS rates. The principle of calculating R_d is to divide the net tracer uptake (i.e. by the Fick principle) by the precursor pool (i.e. enrichment of tracer in the arterialised venous supply) to allow conversion between tracer uptake to tracee uptake, and was calculated as follows:

$$(2)\quad R_d = \frac{tracer\ uptake}{E_A} = \frac{[C_A \times E_A - C_V \times E_V] \times BF}{E_A}$$

(where R_d is rate of disappearance (of phenylalanine or leucine) from plasma, E_A is arterialised venous enrichment of labelled phenylalanine or leucine, C_A and C_V are arterialised venous and deep-venous phenylalanine/leucine concentrations, respectively, and BF is blood flow.)

Accounting for the lack of primary metabolism of phenylalanine in skeletal muscle, the R_a of phenylalanine therefore reflects the appearance of phenylalanine in plasma resulting from muscle protein breakdown, and can be calculated as follows:

$$(3)\quad R_a = R_d - NB_{phe}$$

Statistics

All data are expressed as means±SEM. Baseline characteristics between groups were tested using an independent samples *t*-test. Postabsorptive amino acid balance and amino acid rates of disappearance data (calculated as a single value from the mean of the two measurements taken in the postabsorptive state) were analysed with a 2-way repeated measures ANOVA with immobilization (pre- and post- immobilization) as the within-subject factor and diet (CON, HFD) as the between-subject factor. For all other variables data were analysed using 3-way repeated measures ANOVA with immobilization (pre- and post- immobilization) and time (within the experimental test day the response to mixed meal ingestion; applicable to plasma related variables and blood flow) or arm (applicable to forearm muscle aCSA and grip strength) as within-subject factors, and diet (CON vs HFD) as the between-subject factor. In case of a significant three-way interaction, additional two-way Repeated Measures ANOVAs were performed to locate the effect. When a significant two-way interaction was detected in any of the analyses, a Bonferroni post hoc test was applied to locate the individual differences. Statistical data analysis was performed using SPSS version 25.0 (IBM Corp, Armonk, NY, USA). Statistical significance was set at $P<0.05$.

Results

Participants' characteristics and dietary intervention

Participants' anthropometric and health related characteristics, and details of the habitual and prescribed diets are presented in **Tables 1** and **2**, respectively. There were no differences between CON and HFD in any participant characteristic. With respect to diet, habitual diets were similar between groups (P>0.05 for all parameters) and both groups decreased protein intake (both absolute, relative to body mass, and as % of total energy intake; all P<0.01) and increased absolute carbohydrate intake (P<0.05) during the controlled experimental period. Significant time x diet interaction effects were observed such that the HFD group, but not CON group, increased their total energy (P<0.001) intake, attributable to an approximate 150% increase in fat intake (P<0.001). Fibre intake also increased (P<0.05) in the HFD group only. The increase in fat intake in HFD led to significant decreases in the % contribution to total energy intake of protein (P<0.01) and carbohydrate (P<0.001) both relative to habitual and CON during the experimental period.

Forearm muscle mass and handgrip strength

Forearm muscle anatomical cross-sectional area (aCSA) (**A**) and forearm maximal handgrip strength (**B**) data pre- and post-immobilization are shown in **Figure 1**. Forearm muscle aCSA was 7.6±1.4% (CON; P<0.01) and 6.0±1.7% (HFD; P<0.01) greater in the dominant control (pre-immobilization values of 4222±207 and 4241±205 mm^2 in CON and HFD, respectively) compared with the non-dominant immobilised (pre-immobilization values of 3901±196 and 3976±223 mm^2 in CON and HFD, respectively) arm (P<0.001). However, no effects of immobilization (P=0.60), diet (P=0.17) or any interactions (all P>0.05) were observed. Handgrip strength was also greater (4.3±1.9 [CON] and 1.8±3.7% [HFD]) in the control (pre-immobilization values of 42.2±3.3 and 42.5±2.8 kg in CON and

HFD, respectively) compared with the immobilised (pre-immobilization values of 40.0±2.8 and 41.1±1.9 kg in CON and HFD, respectively) arm (P<0.0001). A significant immobilization x arm interaction was observed (P<0.001) such that the control arm remained unchanged (P=0.117) but handgrip strength in the immobilised arm decreased (P<0.001) following immobilization and by a similar extent in both groups (by 16.6±3.3 and 12.7±3.5% in the CON and HFD, respectively), though no effects of diet (P=0.72) or any interactions were detected (all P>0.05).

Serum insulin concentrations

Figure 2 depicts arterialised venous serum insulin concentrations in the postabsorptive state and in response to the ingestion of a mixed meal pre- and post- immobilization. Some of these serum insulin data have, in part been published previously (12). Postabsorptive serum insulin concentrations were higher in the HFD compared with CON group (P<0.05), increased following immobilization (from 12±1 to 13±1 $mU \cdot L^{-1}$ in CON and from 14±2 to 19±2 $mU \cdot L^{-1}$ in HFD; P<0.05) but did not change divergently across groups (diet x immobilization interaction; P=0.18). Mixed meal ingestion increased serum insulin concentrations (effect of meal ingestion; P<0.001) to an equivalent degree in CON (peaking pre-immobilization at 97±14 $mU \cdot L^{-1}$ and post-immobilization at 104±14 $mU \cdot L^{-1}$) and HFD (peaking pre-immobilization at 96±12 $mU \cdot L^{-1}$ and post-immobilization at 109±12 $mU \cdot L^{-1}$) with peak concentrations always observed 40 min post meal ingestion, and staying elevated above postabsorptive values for the entire postprandial period. While serum insulin concentrations during the 3 h experimental period were higher post- compared with pre-immobilization (P<0.05) there was no difference between groups (effect of diet; P=0.15) nor any interactions detected (all P>0.05). When expressing as the total postprandial area under the curve, the serum insulin response to mixed meal ingestion was also greater post- compared with pre-immobilization (P<0.05), but was unaffected by diet (P=0.18) without any interaction (P=0.42).

Plasma amino acid concentrations

Arterialised venous plasma phenylalanine and leucine concentrations in the postabsorptive state and in response to mixed meal ingestion are shown in **Figure 3**. Mixed meal ingestion increased arterialised plasma phenylalanine concentrations (P<0.001) to an equivalent degree in both groups (**A** and **B**, effect of diet; P=0.34) with comparable responses pre- and post-immobilization (effect of immobilization; P=0.52) and no divergent effects between groups (all interactions; P>0.05). Specifically, in the CON group, meal ingestion increased plasma phenylalanine concentrations from postabsorptive levels of 43±1 to a peak of 64±4 μmol·L^{-1} after 40 min, and from 47±2 to a peak of 64±2 μmol·L^{-1} after 40 min pre- and post-immobilization, respectively. In the HFD group, meal ingestion increased plasma phenylalanine concentrations from postabsorptive levels of 49±4 to a peak of 68±6 μmol·L^{-1} after 40 min, and from 46±4 to a peak of 65±5 μmol·L^{-1} after 60 min pre- and post- immobilization, respectively. In both groups, plasma phenylalanine concentrations had not returned to postabsorptive levels by the end of the postprandial period (P>0.05).

Mixed meal ingestion increased plasma leucine concentrations (P<0.001) to an equivalent degree in both groups (**C** and **D**; effect of diet; P=0.32) with comparable responses pre- and post-immobilization (P=0.14) and no divergent effects between groups (all interactions; P>0.05). In the CON group, meal ingestion increased plasma leucine concentrations from postabsorptive levels of 148±6 to a peak of 251±17 μmol·L^{-1} after 40 min, and from 155±5 to a peak of 244±12 μmol·L^{-1} after 40 min pre- and post- immobilization, respectively. In the HFD group, meal ingestion increased plasma leucine concentrations from postabsorptive levels of 158±8 to a peak of 248±12 μmol·L^{-1} after 40 min, and from 149±7 to a peak of 244±10 μmol·L^{-1} after 40 min pre- and post-immobilization, respectively. In both groups, plasma leucine concentrations had not returned to postabsorptive levels by the end of the postprandial period (P>0.05).

Arterialised venous-deep venous (AV-V) amino acid kinetics

Some of the data relating to brachial artery blood flow have been utilised in a previous publication (12). Brachial artery blood flow increased with mixed meal ingestion (P<0.001), with no difference between groups (effect of diet; P=0.72) and equivalently pre- and post-immobilization (effect of immobilization; P=0.87) with no interactions (all P>0.05). The increase in blood flow was significantly greater than postabsorptive values (~60-70 mL·min^{-1}) from 100-140 min of the postprandial period.

Figure 4 illustrates the dynamic response of forearm phenylalanine (**A** and **B**) and leucine (**C** and **D**) net balances in response to mixed meal ingestion. In the postabsorptive state, forearm phenylalanine net balance showed a tendency to be lower post- compared with pre- immobilization (P=0.07), but was not affected by diet (P=0.51) or a diet x immobilization interaction (P=0.21). Meal ingestion switched forearm phenylalanine net balance from negative (-25±6 and -29±6 µmol·min^{-1}·100 mL^{-1} forearm volume in CON and -22±7 and -43±9 µmol·min^{-1}·100 mL forearm volume^{-1} in HFD, pre- and post- immobilization, respectively) to positive (effect of meal ingestion; P<0.01), an effect which pre-immobilization peaked at 25±4 µmol·min·100 mL forearm volume^{-1} after 40 min in CON and 25±20 µmol·min^{-1}·100 mL forearm volume^{-1} after 120 min in HFD, before returning to negative between 60 and 140 min into the postprandial period. There was a tendency (P=0.06) for forearm phenylalanine net balance to be reduced post- compared with pre- immobilization, such that mixed meal ingestion did not (or minimally so) achieve positive forearm phenylalanine balance at any point in the postprandial phase post-immobilization. Although there were no group differences (effect of diet; P=0.61), a significant immobilization x meal ingestion x diet interaction (P<0.05) and a tendency for an immobilization x meal interaction (P=0.10) was observed, with no other interactions (all P>0.05). This 3-way interaction manifested as a time effect (P<0.01) and immobilization x meal ingestion interaction (P<0.001) in the HFD group only. When expressing forearm phenylalanine net

balance as the 3 h post-prandial AUC, a tendency for an effect of immobilization (P=0.072) was observed, with no effect of diet (P=0.48) or immobilization x diet interaction (P=0.39).

In the postabsorptive state, forearm leucine net balance was lower post- compared with pre-immobilization (P=0.05) but was not affected by diet (P=0.84) or a diet x immobilization interaction (P=0.28). Mixed meal ingestion switched forearm leucine net balance from negative (-59±33 and -82±15 μmol·min^{-1}·100 mL forearm volume^{-1} in CON and -39±16 and -113±25 μmol·min^{-1}·100 mL forearm volume^{-1} in HFD, pre- and post- immobilization, respectively) to positive (effect of meal ingestion; P<0.001) an effect which, pre-immobilization peaked at 184±133 and 132±34 μmol·min^{-1}·100 mL forearm volume^{-1} after 40 min in CON and HFD groups, respectively, before returning negative between 60 and 140 min into the postprandial period. There was a tendency (effect of immobilization; P=0.07) for a lower forearm leucine net balance post- compared with pre-immobilization, such that mixed meal ingestion did not (or minimally so) achieve positive forearm leucine balance post-immobilization. Although there were no group differences (effect of diet; P=0.39), a significant immobilization x meal ingestion x diet interaction (P<0.001) and a tendency for an immobilization x meal ingestion interaction (P=0.06) were observed, with no other interactions (all P>0.05). This 3-way interaction manifested as tendencies for meal ingestion (P=0.05) and immobilization x meal ingestion (P=0.06) interactions in the CON group, and an effect of meal ingestion (P<0.01) and immobilization x meal ingestion interaction (P<0.001) in the HFD group. When expressing forearm leucine net balance as the 3 h postprandial AUC, a tendency for an effect of immobilization (P=0.072) was observed such that total postprandial AUC was lower following immobilization, with no effect of diet (P=0.27) or immobilization x diet interaction (P=0.61).

Amino acid tracer dynamics

Figure 5 depicts the arterialised plasma L-[*ring*-$^{2}H_5$]phenylalanine (**A** and **B**) and [1-^{13}C]leucine (**C** and **D**) enrichments in the CON and HFD groups, respectively. Primed continuous tracer infusion increased arterialised plasma L-[*ring*-$^{2}H_5$]phenylalanine and [1-^{13}C]leucine enrichments from virtually zero background enrichments to ~7 and ~9 mole percent excess (MPE) in CON and ~6 and ~8 MPE in HFD. Systemic entry of unlabelled amino acids following mixed meal ingestion (i.e. *t*=0) decreased arterialised plasma L-[*ring*-$^{2}H_5$]phenylalanine and [1-^{13}C]leucine enrichments to ~6 and ~7 mole percent excess (MPE) in CON and ~5 and ~6 MPE in HFD, respectively (effects of mixed meal ingestion; all *P*<0.001), with no effects of immobilization, diet or any interactions. The initial (20 min) decrease in arterialised plasma L-[*ring*-$^{2}H_5$]phenylalanine and [1-^{13}C]leucine enrichments following meal ingestion then remained in steady state for the remainder of the postprandial period. Due to the systemic and steady state delivery of the tracers, venous plasma L-[*ring*-$^{2}H_5$]phenylalanine and [1-^{13}C]leucine enrichments followed precisely the same patterns and magnitude as arterialised enrichments, with the exception that enrichments returned to fasted levels prior to the end of the postprandial period (by 140 and 160 min for L-[*ring*-$^{2}H_5$]phenylalanine and [1-^{13}C]leucine, respectively).

Figure 6 illustrates the forearm phenylalanine (**A** and **B**) and leucine (**C** and **D**) rates of disappearance (R_d) in response to the ingestion of a mixed meal. Postabsorptive forearm phenylalanine R_d (18±12 and 22± in CON and 38±5 and 34±6 μmol·min^{-1}·100 mL forearm volume^{-1} in HFD, pre- and post-immobilization, respectively) were not affected throughout the study (effects of: immobilization, *P*=0.98; diet, *P*=0.13; immobilization x diet interaction, *P*=0.67). Forearm phenylalanine R_d tended to increase with meal ingestion (*P*=0.10) peaking at 48±12 (20 min) and 28±12 (40 min) in CON and 99±24 (120 min) and 81±17 (20 min) μmol·min^{-1}·100 mL forearm volume^{-1} in HFD pre- and post-immobilization, respectively. Forearm phenylalanine R_d were overall significantly higher in the HFD group (effect of diet; *P*<0.05), with no effect of immobilization (*P*=0.37) or any interactions evident

(all P>0.05). In agreement, forearm phenylalanine R_d expressed as postprandial AUC exhibited a significant effect of diet (P<0.05), but not immobilization (P=0.26) or immobilization x diet interaction (P=0.26).

Postabsorptive forearm leucine R_d (87±38 and 115±24 µmol·min^{-1} in CON and 141±24 and 126±25 µmol·min^{-1} in HFD, pre- and post- immobilization, respectively) were not affected throughout the study (effects of: immobilization, P=0.81; diet, P=0.32; immobilization x diet interaction, P=0.43). Forearm leucine R_d significantly increased with mixed meal ingestion (P<0.05). In the CON group this resulted in peak responses of a 191% increase (to 254±60 µmol·min^{-1}) after 100 min and a 41% increase (to 162±41 µmol·min^{-1}) 40 min after mixed meal ingestion pre- and post- immobilization, respectively. In the HFD group peak responses of a 183% increase (to 339±51 µmol·min^{-1}) after 120 min and 152% (to 318±54 µmol·min^{-1}) 20 min after mixed meal ingestion were observed pre- and post- immobilization, respectively. Forearm leucine R_d were not different between groups (effect of diet; P=0.13) or immobilization (P=0.50), though significant immobilization x time (P<0.05) and immobilization x time x diet (P<0.05) interactions were detected, with other interactions not significant (both P>0.05). The 3-way interaction manifested as immobilization x meal ingestion interactions in both the CON (P<0.05) and HFD (P<0.05), and meal ingestion effects in the CON (P<0.05) and HFD (P<0.05) pre, but not post (both P>0.05) immobilization. As a result the increase in forearm leucine R_d was blunted post- compared with pre-immobilization and to a comparable extent between CON and HFD. Despite the higher peak values post-immobilization in the HFD group, the average postprandial leucine R_d was ~25% lower in HFD compared with CON group (P<0.05) though this did not translate to differing forearm leucine R_d expressed as postprandial AUC across groups (all main effects; P>0.05).

Postabsorptive forearm phenylalanine rates of appearance (R_a) (**Figure 7**) were unaffected by immobilization (P=0.17), diet (P=0.14) or any immobilization x diet interaction (P=0.60). Forearm phenylalanine R_a also exhibited no main effects of mixed meal ingestion (P=0.12) nor any other main

effects in the postprandial state (all $P>0.05$), nor any significant effects when analysing postprandial AUC data (all $P>0.05$). Postabsorptive forearm leucine R_a (data not shown) were unaffected by immobilization, diet or any immobilization x diet interaction (all $P>0.05$). Forearm leucine R_a also exhibited no main effects or interactions (all $P>0.05$); however, a significant immobilization x meal ingestion interaction was detected ($P<0.05$) such that forearm leucine R_a increased post- compared with pre- immobilization.

Discussion

The present study demonstrates for the first time that muscle disuse leads to a decrease in postabsorptive and postprandial net amino acid accretion within the forearm (i.e. a less positive amino acid balance), with the postprandial state being associated with an impairment in muscle amino acid uptake (i.e. amino acid rate of disappearance into the forearm) but not changes in muscle protein breakdown (i.e. phenylalanine rate of appearance from the forearm). Additionally, while the availability of excess dietary lipids via provision of a high-fat, hypercaloric diet did not impact on muscle amino acid uptake or breakdown, it did exacerbate the negative impact of disuse on the ability of food intake to promote a positive amino acid balance across the forearm. Finally, although one week of immobilization reduced forearm muscle strength, no loss of forearm muscle mass was detected.

Declining postabsorptive and postprandial (i.e. anabolic resistance) muscle protein synthesis (MPS) rates are widely regarded as key mechanisms responsible for muscle disuse atrophy (4-6). Since postabsorptive muscle protein breakdown (MPB) rates do not appear to change with disuse (45), it is generally assumed that declines in postabsorptive and postprandial MPS rates manifest as a chronic reduction in muscle protein balance and thus net protein loss. However, the impact of disuse on muscle amino acid net balance had not been confirmed to date. Here, we show that disuse reduced postabsorptive net amino acid balance across the forearm (by 16% and 39% in the eucaloric control group when considering phenylalanine or leucine net balances, respectively), with no additional effect of dietary lipid oversupply (Figure 4). Our data are in line with declining MPS rates as the primary driver of reduced muscle amino acid balance, since postabsorptive forearm protein breakdown (Figure 7) did not change following immobilization. Under conditions of habitual physical activity (i.e. pre-immobilization), we report mixed meal ingestion resulted in circulating amino acid and insulin responses (Figures 2-3) capable of switching forearm amino acid turnover from net loss to net accretion. Despite equivalent postprandial systemic amino acid supply post-immobilization,

the postprandial switch from negative to positive net amino acid balance was impaired. In fact, following disuse participants were unable to mount a positive postprandial net amino acid balance across the forearm such that a ~40% reduction in postprandial phenylalanine net balance (expressed as total postprandial area under the curve) was observed. While the absence of muscle tissue samples precluded us from measuring MPS rates directly, the parallel lack of change in postprandial phenylalanine release form the forearm (i.e. muscle protein breakdown) implies an impaired ability to mount a postprandial net positive protein balance was driven by a reduced stimulation of MPS rates (i.e. anabolic resistance). In line with previous work we did not observe a decline in muscle mass of the forearm (Figure 1) which, in contrast to the rapid disuse atrophy evident in leg muscles (1), typically takes more than one week to manifest in the forearm (46-48). Accordingly, our data supports previous assumptions that the development of muscle anabolic resistance is a key mechanism preceding and responsible for subsequent muscle loss (49). Muscle strength during disuse declined despite the lack of measurable atrophy, corroborating previous findings that function declines more rapidly than mass (50), presumably due to rapid neuromuscular deconditioning (49, 51).

Since anabolic resistance was induced under energy balanced conditions, the mechanisms seem to be driven by the withdrawal of muscle contraction *per se*. However, we have recently shown that disuse-induced insulin resistance is associated with an increased non-esterified fatty acid (NEFA) balance across the forearm, even under energy-balanced conditions (12). This opens up the intriguing possibility that altered local lipid trafficking (e.g. increased muscle or adipose NEFA uptake, reduced adipose release and/or altered muscle/adipose crosstalk) may underpin the development of anabolic resistance. To test the role of lipid availability in the perturbation of muscle amino acid metabolism, we hypothesised that provision of a high-fat diet would exacerbate the impact of disuse upon postprandial forearm amino acid balance. In line, we observed a further diminishment in the ability of mixed meal ingestion to improve forearm net amino acid balance under high dietary fat conditions. While we have repeatedly shown that 5-7 days of disuse does not

result in frank muscle lipid accumulation (52, 53), we have recently reported that even one day of disuse under eucaloric conditions leads to transcriptional changes suggestive of increased lipid intermediate (e.g. diacylglycerol) synthesis (54), which translates to muscle accumulation of certain diacylglycerol subspecies within one week of bedrest (52). It is therefore plausible that our current data can be explained by intracellular inhibitory effects of such lipid metabolites on myocellular anabolic signalling (16, 18, 55), which occur with disuse *per se* but are worsened under conditions of dietary lipid oversupply. Interestingly, our amino acid balance calculations suggest that if immobilization had persisted until muscle loss was detectable, we would have observed a 20-30% greater rate of muscle atrophy in the high-fat overfed group, which is in line with previous work imposing hypercaloric diets over more prolonged periods of disuse (56, 57).

A primary goal of this study was to establish whether anabolic resistance was associated with reduced muscle amino acid uptake, thereby providing evidence for an extra- rather than intra-cellular mechanism (i.e. a blunting of the 'signal' rather than 'sensing'). By applying a dual stable isotope labelled amino acid approach in parallel with the AV-V forearm balance method, we were able to quantify the impact of disuse on dynamic rates of muscle amino uptake *in vivo*. Expectedly, the rise in arterial supply of amino acids following mixed meal ingestion stimulated rates of muscle amino acid uptake (Figure 6). In line with our hypothesis, we report that disuse impaired the rise in postprandial forearm muscle amino acid uptake. This result was most clear when tracing plasma leucine disappearance into forearm muscle tissue where a ~20% reduction in peak postprandial leucine uptake was observed following immobilization. This occurred despite brachial artery leucine delivery (i.e. flow multiplied by concentration) to the muscle remaining constant, indicating the impairment resides at the level of microvascular flow/muscle perfusion and/or cellular amino acid uptake. In support, it has previously been proposed that age-related insulin resistance of protein metabolism exists, since insulin stimulated endothelial function, muscle perfusion and muscle transcriptional/translational responses of amino acid transporters are all impaired with age, but overcome by hyperinsulinaemic conditions or a single bout of exercise (58-60). Although recent data

demonstrate high-fat overfeeding also results in impaired muscle endothelial nitric oxide synthase phosphorylation and content in terminal arterioles (61), here we report that postprandial muscle amino acid uptake was impaired post-immobilization to a similar extent regardless of the dietary condition. In keeping with studies showing the stimulatory role of exercise (36), our data therefore indicate that muscle contraction *per se* is the primary regulator of muscle amino acid uptake.

Although the association of reduced postprandial muscle amino acid uptake under energy balanced conditions indicates a potential mechanistic link to reduced MPS (which could conceivably exist as a feedforward or feedback loop), the aggravating effect of lipid on muscle amino acid balance only, suggests independent mechanisms regulating anabolic sensitivity. That is, lipid oversupply appears to exert its control only via intracellular mechanisms. Thus, it could be speculated that any lipid induced impairments in myocellular amino acid metabolism (reduced/impaired oxidation and/or reduced ribosomal incorporation into polypeptide chains via the mTOR signalling pathway with no consequent reduction of muscle amino acid uptake, as we observed), would result in the accumulation of intracellular (branched chain) amino acids and consequent overspill (over time) into the circulation. Indeed, intracellular accumulation of branched chain amino acids has been reported following a period of disuse (5), and we observed an increased muscle leucine release in the present data. This could have important implications as there is a growing literature base demonstrating elevated circulating branched chain amino acids (and their metabolites) being implicated in the onset of insulin resistance and development of type-2 diabetes (62, 63). Therefore, the presence, relevance and relation to lipid metabolism, of elevated intracellular branched chain amino acids in various compromised populations warrants future investigation.

The present work demonstrates that disuse reduces postabsorptive and postprandial muscle amino acid net balances (i.e. forearm net amino acid accretion), with the latter exacerbated by dietary lipid oversupply. Disuse induced alterations in muscle amino acid handling were associated with reduced postprandial amino acid uptake in the absence of any changes in muscle protein breakdown. Our

data imply that impaired muscle amino acid uptake may be a key mechanistic factor in the aetiology of disuse induced anabolic resistance and that disuse and lipid supply may regulate anabolic sensitivity at differing levels of physiology.

References

1. **Kilroe S, Fulford J, Jackman S, van Loon L, Wall B** 2019 Temporal Muscle-Specific Disuse Atrophy during One Week of Leg Immobilization. Med Sci Sports Exerc [Epub ahead of print]

2. **Dirks M, Wall B, van de Valk B, Holloway T, Holloway G, Chabowski A, Goossens G, van Loon L** 2016 One week of bed rest leads to substantial muscle atrophy and induces whole-body insulin resistance in the absence of skeletal muscle lipid accumulation. Diabetes 65:2862-2875

3. **Booth F, Gollnick P** 1983 Effects of disuse on the structure and function of skeletal muscle. Med Sci Sports Exerc 15:415-420

4. **Wall B, Dirks M, Snijders T, Van Dijk J, Fritsch M, Verdijk L, van Loon L** 2016 Short-term muscle disuse lowers myofibrillar protein synthesis rates and induces anabolic resistance to protein ingestion Am J Physiol 310:137-147

5. **Glover E, Phillips S, Oates B, Tang J, Tarnopolsky M, Selby A, Smith K, Rennie M** 2008 Immobilization induces anabolic resistance in human myofibrillar protein synthesis with low and high dose amino acid infusion. J Physiol 586:6049-6061

6. **Drummond M, Dickinson J, Fry C, Walker D, Gundermann D, Reidy P, Timmerman K, Markofski M, Paddon-Jones D, Rasmussen B, Volpi E** 2012 Bed rest impairs skeletal muscle amino acid transporter expression, mTORC1 signaling, and protein synthesis in response to essential amino acids in older adults. Am J Physiol Endocrinol Metab 302:1113-1122

7. **Cuthbertson D, Smith K, Babraj J, Leese G, Waddell T, Atherton P, Wackerhage H, Taylor P, Rennie M** 2005 Anabolic signaling deficits underlie amino acid resistance of wasting, aging muscle. FASEB J 19:422-424

8. **Dickinson J, Rasmussen B** 2013 Amino acid transporters in the regulation of human skeletal muscle protein metabolism. Curr Opin Clin Nutr Metab Care 16:638-644

9. **Drummond M, Glynn E, Fry C, Timmerman K, Volpi E, Rasmussen B** 2010 An increase in essential amino acid availability upregulates amino acid transporter expression in human skeletal muscle. Am J Physiol 298:1011-1018

10. **Beals J, Sukiennik R, Nallabelli J, Emmons R, van Vliet S, Young J, Ulanov A, Li Z, Paluska S, De Lisio M, Burd N** 2016 Anabolic sensitivity of postprandial muscle protein synthesis to the ingestion of a protein-dense food is reduced in overweight and obese young adults. Am J Clin Nutr 104:1014-1022

11. **Dirks M, Stephens F, Jackman S, Galera Gordo J, Machin D, Pulsford R, van Loon L, Wall B** 2018 A single day of bed rest, irrespective of energy balance, does not affect skeletal muscle gene expression or insulin sensitivity. Exp Physiol 103:860-875

12. **Dirks M, Wall B, Otten B, Cruz A, Dunlop M, Barker A, Stephens F** 2019 High-fat overfeeding does not exacerbate rapid changes in forearm glucose and fatty acid balance during immobilization. J Clin Endocrinol Metab [Epub ahead of print]

13. **Murton A, Marimuthu K, Mallinson J, Selby A, Smith K, Rennie M, Greenhaff P** 2015 Obesity Appears to Be Associated With Altered Muscle Protein Synthetic and Breakdown Responses to Increased Nutrient Delivery in Older Men, but Not Reduced Muscle Mass or Contractile Function. Diabetes 64:3160-3171

14. **Guillet C, Delcourt I, Rance M, Giraudet C, Walrand S, Bedu M, Duche P, Boirie Y** 2009 Changes in basal and insulin and amino acid response of whole body and skeletal muscle proteins in obese men. J Clin Endocrinol Metab 94:3044-3050

15. **Smeuninx B, Mckendry J, Wilson D, Martin U, Breen L** 2017 Age-Related Anabolic Resistance of Myofibrillar Protein Synthesis Is Exacerbated in Obese Inactive Individuals. J Clin Endocrinol Metab 102:3535-3545

16. **Stephens F, Chee C, Wall B, Murton A, van Loon L, Tsintzas K** 2015 Lipid induced insulin resistance is associated with an impaired skeletal muscle protein synthetic response to amino acid ingestion in healthy young men. Diabetes 64:1615-1620

17. **Anderson S, Gilge D, Steiber A, Previs S** 2008 Diet-induced obesity alters protein synthesis: tissue-specific effects in fasted versus fed mice. Metabolism 57:347-354

18. **Masgrau A, Mishellany-Dutour A, Murakami H, Beaufrère A, Walrand S, Giraudet C, Migné C, Gerbaix M, Metz L, Courteix D, Guillet C, Boirie Y** 2012 Time-course changes of muscle protein synthesis associated with obesity-induced lipotoxicity. J Physiol 590:5199-5210

19. **Kouw I, van Dijk J, Horstman A, Kramer I, Goessens J, van Dielen F, Verdijk L, van Loon L** 2019 Basal and Postprandial Myofibrillar Protein Synthesis Rates Do Not Differ between Lean and Obese Middle-Aged Men. J Nutr 149:1533-1542

20. **Gorissen S, Burd N, Kramer I, van Kranenburg J, Gijsen A, Rooyackers O, van Loon L** 2017 Co-ingesting milk fat with micellar casein does not affect postprandial protein handling in healthy older men. Clin Nutr 36:429-437

21. **Elliot T, Cree M, Sanford A, Wolfe R, Tipton K** 2006 Milk ingestion stimulates net muscle protein synthesis following resistance exercise. Med Sci Sports Exerc 38:667-674

22. **Tsintzas K, Jones R, Pabla P, Mallinson J, Barrett D, Kim D, Cooper S, Davies A, Taylor T, Chee C, Gaffney C, van Loon L, Stephens F** 2020 Effect of acute and short-term dietary fat ingestion on postprandial skeletal muscle protein synthesis rates in middle-aged, overweight, and obese men. Am J Physiol Endocrinol Metab 318:417-429

23. **IPAQ** 2002 International Physical Activity Questionnaire. In:

24. **Shannon C, Nixon A, Greenhaff P, Stephens F** 2016 Protein ingestion acutely inhibits insulin-stimulated muscle carnitine uptake in healthy young men. Am J Clin Nutr 103:276-282

25. **Gallen I, Macdonald I** 1990 Effect of two methods of hand heating on body temperature, forearm blood flow, and deep venous oxygen saturation. Am J Physiol 259:639-643

26. **Gallen IW, Macdonald IA** 1990 Effect of two methods of hand heating on body temperature, forearm blood flow, and deep venous oxygen saturation. Am J Physiol 259:E639-643

27. **Andres R, Zierler KL, Anderson HM, Stainsby WN, Cader G, Ghrayyib AS, Lilienthal JL, Jr.** 1954 Measurement of blood flow and volume in the forearm of man; with notes on the theory of indicator-dilution and on production of turbulence, hemolysis, and vasodilatation by intra-vascular injection. J Clin Invest 33:482-504

28. **Hoskins PR** 1990 Measurement of arterial blood flow by Doppler ultrasound. Clin Phys Physiol Meas 11:1-26

29. **Nutritics** In: Nutritics Ltd

30. **Henry C** 2005 Basal metabolic rate studies in humans: measurement and development of new equations. Public Health Nutr 8:1133-1152

31. **Wolfe RR, Chinkes DL** 2005 Isotope tracers in metabolic research: principles and practice of kinetic analysis: John Wiley & Sons

32. **Bornø A, Hulston CJ, van Hall G** 2014 Determination of human muscle protein fractional synthesis rate: an evaluation of different mass spectrometry techniques and considerations for tracer choice. Journal of Mass Spectrometry 49:674-680

33. **Jackson R, Hamling J, Sim B, Hawa M, Blix P, Nabarro J** 1987 Peripheral lactate and oxygen metabolism in man: the influence of oral glucose loading. Metabolism 36:144-150

34. **Pozefsky T, Tancredi R, Moxley R, Dupre J, Tobin J** 1976 Effects of brief starvation on muscle amino acid metabolism in nonobese man. J Clin Invest 57:444-449

35. **Wolfe R, Chinkes D** 2005 Isotope tracers in metabolic research. Wiley-Liss New York

36. **Biolo G, Maggi S, Williams B, Tipton K, Wolfe R** 1995 Increased rates of muscle protein turnover and amino acid transport after resistance exercise in humans. Am J Physiol 268:514-520

37. **Biolo G, Fleming R, Maggi S, Wolfe R** 1995 Transmembrane transport and intracellular kinetics of amino acids in human skeletal muscle. Am J Physiol 268:75-84

38. **Forster H, Dempsey J, Thomson J, Vidruk E, DoPico G** 1972 Estimation of arterial PO2, PCO2, pH, and lactate from arterialized venous blood. J Appl Physiol 32:134-137

39. **Macdonald I** 1999 Arterio-venous differences to study macronutrient metabolism: introduction and overview. Proc Nutr Soc 58:871-875

40. **Zierler K, Rabinowitz D** 1963 Roles of inulins and growth hormone, based on studies of forearm metabolism in man. Medicine (Baltimore) 42:385-402

41. **Zierler K** 1961 Theory of the use of arteriovenous concentration differences for measuring metabolism in steady and non-steady states. . J Clin Invest 40:2111-2125

42. **Hoskins P** 1990 Measurement of arterial blood flow by Doppler ultrasound. Clin Phys Physiol Meas 11:1-26

43. **Wall B, Hamer H, de Lange A, Kiskini A, Groen B, Senden J, Gijsen A, Verdijk L, van Loon L** 2013 Leucine co-ingestion improves post-prandial muscle protein accretion in elderly men. Clin Nutr 32:412-419

44. **Wall B, Snijders T, Senden J, Ottenbros C, Gijsen A, Verdijk L, van Loon L** 2013 Disuse impairs the muscle protein synthetic response to protein ingestion in healthy men. J Clin Endocrinol Metab 98:4872-4878

45. **Symons T, Sheffield-Moore M, Chinkes D, Ferrando A, Paddon-Jones D** 2009 Artificial gravity maintains skeletal muscle protein synthesis during 21 days of simulated microgravity. J Appl Physiol 107:34-38

46. **Farthing J, Krentz J, Magnus C** 2009 Strength training the free limb attenuates strength loss during unilateral immobilization. J Appl Physiol 106:830-836

47. **MacDougall J, Ward G, Sale D, Sutton J** 1977 Biochemical adaptation of human skeletal muscle to heavy resistance training and immobilization. J Appl Physiol 43:700-703

48. **Seki K, Taniguchi Y, Narusawa M** 2001 Effects of joint immobilization on firing rate modulation of human motor units. J Physiol 530:507-519

49. **Wall B, van Loon L** 2012 Nutritional strategies to attenuate muscle disuse atrophy. Nutrition Reviews 71:195-208

50. **Kawakami Y, Akima H, Kubo K, Muraoka Y, Hasegawa H, Kouzaki M, Imai M, Suzuki Y, Gunji A, Kanehisa H, Fukunaga T** 2001 Changes in muscle size, architecture, and neural activation after 20 days of bed rest with and without resistance exercise. Eur J Appl Physiol Occup Physiol 84:7-12

51. **Deschenes M, Giles J, McCoy R, Volek J, Gomez A, Kraemer W** 2002 Neural factors account for strength decrements observed after short-term muscle unloading. Am J Physiol Regul Integr Comp Physiol 282:578-583

52. **Dirks ML, Wall BT, van de Valk B, Holloway TM, Holloway GP, Chabowski A, Goossens GH, van Loon LJ** 2016 One Week of Bed Rest Leads to Substantial Muscle Atrophy and Induces Whole-Body Insulin Resistance in the Absence of Skeletal Muscle Lipid Accumulation. Diabetes 65:2862-2875

53. **Wall BT, Dirks ML, Snijders T, Stephens FB, Senden JM, Verscheijden ML, van Loon LJ** 2015 Short-term muscle disuse atrophy is not associated with increased intramuscular lipid deposition or a decline in the maximal activity of key mitochondrial enzymes in young and older males. Exp Gerontol 61:76-83

54. **Dirks ML, Stephens FB, Jackman SR, Gordo JG, Machin D, Pulsford RM, van Loon LJ, Wall BT** 2018 A single day of bed rest, irrespective of energy balance, does not affect skeletal muscle gene expression or insulin sensitivity. Exp Physiol

55. **Guillet C, Masgrau A, Walrand S, Boirie Y** 2012 Impaired protein metabolism: interlinks between obesity, insulin resistance and inflammation. Obes Rev suppl 2

56. **Biolo G, Agostini F, Simunic B, Sturma M, Torelli L, Preiser J, Deby-Dupont G, Magni P, Strollo F, di Prampero P, Guarnieri G, Mekjavic I, Pisot R, Narici M** 2008 Positive energy balance is associated with accelerated muscle atrophy and increased erythrocyte glutathione turnover during 5 wk of bed rest. Am J Clin Nutr 88:950-958

57. **Rejc E, di Prampero P, Lazzer S, Grassi B, Simunic B, Pisot R, Antonutto G, Narici M** 2015 Maximal explosive power of the lower limbs before and after 35 days of bed rest under different diet energy intake. Eur J Appl Physiol 115:429-436

58. **Fujita S, Glynn E, Timmerman K, Rasmussen B, Volpi E** 2009 Supraphysiological hyperinsulinaemia is necessary to stimulate skeletal muscle protein anabolism in older adults: evidence of a true age-related insulin resistance of muscle protein metabolism. Diabetologia 52:1889-1898

59. **Fujita S, Rasmussen B, Cadenas J, Drummond M, Glynn E, Sattler F, Volpi E** 2007 Aerobic exercise overcomes the age-related insulin resistance of muscle protein metabolism by improving endothelial function and Akt/mammalian target of rapamycin signaling. Diabetes 56:1615-1622

60. **Fujita S, Rasmussen B, Cadenas J, Grady J, Volpi E** 2006 Effect of insulin on human skeletal muscle protein synthesis is modulated by insulin-induced changes in muscle blood flow and amino acid availability. Am J Physiol Endocrinol Metab 291:745-754

61. **Parry S, Turner M, Woods R, James L, Ferguson R, Cocks M, Whytock K, Strauss J, Shepherd S, Wagenmakers A, van Hall G, Hulston C** 2019 High-fat overfeeding impairs peripheral glucose metabolism and muscle microvascular eNOS Ser1177 phosphorylation. J Clin Endocrinol Metab [Epub ahead of print]

62. **Newgard C, An J, Bain J, Muehlbauer M, Stevens R, Lien L, Haqq A, Shah S, Arlotto M, Slentz C, Rochon J, Gallup D, Ilkayeva O, Wenner B, Yancy W, Eisenson H, Musante G, Surwit R, Millington D, Butler M, Svetkey L** 2009 A branched-chain amino acid-related metabolic signature that differentiates obese and lean humans and contributes to insulin resistance. Cell Metab 9:311-326

63. **Wang T, Larson M, Vasan R, Cheng S, Rhee E, McCabe E, Lewis G, Fox C, Jacques P, Fernandez C, O'Donnell C, Carr S, Mootha V, Florez J, Souza A, Melander O, Clish C, Gerszten R** 2011 Metabolite profiles and the risk of developing diabetes. Nat Med 17:448-453

Tables

Table 1: Participants' characteristics

Parameter	CON	HFD	*P* value
Age (y)	24±2	24±2	0.89
Body mass (kg)	74±5	72±4	0.69
Height (cm)	177±2	179±2	0.63
BMI ($kg \cdot m^{-2}$)	24±1	23±1	0.43
Body fat (% of body mass)	14 ±4	12±3	0.60
Lean mass (kg)	57±7	63±3	0.47
Systolic blood pressure (mm Hg)	117±3	120±2	0.39
Diastolic blood pressure (mm Hg)	65±2	66±2	0.69

Values represent means ± SEM. BMI, body mass index. P value represents t-test comparison across groups for each parameter.

Table 2: Dietary intake

Nutritional parameter	CON		HFD	
	Habitual	Experimental	Habitual	Experimental
Energy (MJ·d^{-1})	11.5±0.9	11.8±0.3	10.6±0.9	18.3±0.6*#
Protein (g·kg bm^{-1}·d^{-1})	1.71±0.16	1.21±0.00*	1.68±0.20	1.20±0.00*
Protein (g·d^{-1})	123±13	89±5*	120±14	88±4*
Carbohydrates (g·d^{-1})	310±22	365±9*	257±18	385±13*
Fat (g·d^{-1})	104±12	103±3	108±13	269 ±9 *#
Fibres (g·d^{-1})	29±4	30±1	21±1	36±1*#
Protein (En%)	18±1	13±0 *	19±1	8±0*#
Carbohydrate (En%)	46±1	52±1*	42±2	35±0 *#
Fat (En%)	34±2	33±1	38±2	55±0 *#
Fibres (En%)	2±0	2±0	2±0	2±0#

Values (means±SEM) represent parameters of dietary intake from *n*=20 healthy, male volunteers. Self-reported habitual food intake was assessed using 3-day food diaries, while the diet during 7 days of forearm immobilization (experimental) was calculated and provided in its entirety by the research team. During immobilization, participants were fed a fully-controlled eucaloric diet (CON; *n*=11) or a high-fat diet (HFD; n=9) providing 50% excess energy from fat. Abbreviations: bm, body mass; En%, energy percentage; MJ, Mega Joule. * Significantly different from corresponding habitual intake values (P<0.05). **#** Significantly different from corresponding CON value (P<0.05).

Figure legends

Figure 1. Forearm muscle anatomical cross sectional area (aCSA) (**A**) and maximal grip strength (**B**) pre- and post- one week of forearm immobilization in the non-immobilised (free) and immobilised (immob) arms of healthy young men fed a eucaloric control diet (CON; *n*=11) or a high-fat overfeeding diet (HFD; *n*=9) providing 50% excess energy from fat. CON and HFD data were compared within the same statistical test (3-way ANOVA with arm, immobilization and diet as factors). For muscle aCSA, a significant main effect of arm (i.e. free vs immobilised; *P*<0.001; denoted by *), but no effects of immobilization (i.e. pre vs post; *P*=0.60), diet (*P*=0.95) or any interactions (all *P*>0.05) were observed. For maximal grip strength, significant main effects of arm (*P*<0.001; denoted by *) and immobilization (*P*<0.001), and an immobilization x arm interaction (*P*<0.001) were observed, with no effect of diet (*P*=0.72) or any other interactions (all *P*>0.05) observed. $ denotes significantly different from corresponding pre value.

Figure 2. Arterialised venous serum insulin concentrations pre- and post- one week of forearm immobilization in healthy young men fed a eucaloric control diet (**A**; CON; *n*=11) or a high-fat overfeeding diet (**B**; HFD; *n*=9) providing 50% excess energy from fat in the postabsorptive state (0 min) and for a 3 h postprandial period following the ingestion of a mixed meal. CON and HFD data were compared within the same statistical test (3-way ANOVA with immobilization, time and diet as factors). Significant main effects of immobilization (i.e. pre vs post; *P*<0.05) and time (i.e. response to mixed meal ingestion; *P*<0.001) were observed, with no effects of diet (P=0.29) or any interactions (all *P*>0.05).

Figure 3. Arterialised venous plasma phenylalanine (**A** and **B**) and leucine (**C** and D) concentrations pre- and post- one week of forearm immobilization in healthy young men fed a eucaloric control diet (A and C; CON; *n*=11) or a high-fat overfeeding diet (B and D; HFD; *n*=9) providing 50% excess energy from fat in the postabsorptive (0 min) and for a 3 h postprandial period following the ingestion of a mixed meal. CON and HFD data were compared within the same statistical test (3-way ANOVA with immobilization, time and diet as factors) for each amino acid separately. For phenylalanine, a significant main effect of time (i.e. response to mixed meal ingestion; $P<0.001$) was observed, with no effect of diet ($P=0.34$), immobilization (i.e. pre vs post; $P=0.52$) or any interactions (all $P>0.05$). For leucine, a significant main effect of time ($P<0.001$) was observed, with no effect of diet ($P=0.32$), immobilization ($P=0.16$) or any interactions (all $P>0.05$).

Figure 4. Forearm phenylalanine (**A** and **B**) and leucine (**C** and D) net balance pre- and post- one week of forearm immobilization in healthy young men fed a eucaloric control diet (A and C; CON; *n*=11) or a high-fat overfeeding diet (B and D; HFD; *n*=9) providing 50% excess energy from fat in the postabsorptive state (0 min) and for a 3 h postprandial period following the ingestion of a mixed meal. Inset figures represent the postprandial area under the curves (AUC). CON and HFD data were compared within the same statistical test (3-way ANOVA with immobilization, time and diet as factors) for each amino acid separately, with AUC data analysed with 2-way ANOVAs. For forearm phenylalanine net balance, a significant main effect of time (i.e. response to mixed meal ingestion; $P<0.01$) and a tendency for an effect of immobilization (i.e. pre vs post; $P=0.06$) was observed, with no effect of diet ($P=0.61$). A significant immobilization x time x diet interaction ($P<0.05$) and tendency for an immobilization x time interaction ($P=0.10$) was observed, with no other interactions (all $P>0.05$). The 3-way interaction manifested as a time effect ($P<0.01$) and immobilization x time interaction ($P<0.001$) in the HFD group, only. For forearm phenylalanine net balance AUC, a tendency for an effect of immobilization ($P=0.072$; denoted by #) was observed, with no effect of

diet (*P*=0.48) or immobilization x diet interaction (*P*=0.39). For forearm leucine net balance, a significant main effect of time (i.e. response to mixed meal ingestion; *P*<0.001) and a tendency for an effect of immobilization (i.e. pre vs post; *P*=0.07) was observed, with no effect of diet (*P*=0.39). A significant immobilization x time x diet interaction (*P*<0.001) and tendency for an immobilization x time interaction (*P*=0.06) was observed, with no other interactions (all *P*>0.05). The 3-way interaction manifested as tendencies for a time (*P*=0.05) and immobilization x time (*P*=0.06) interaction effects in the CON group, and a time effect (*P*<0.01) and immobilization x time interaction (*P*<0.001) in the HFD group. For forearm leucine net balance AUC, a tendency for an effect of immobilization (*P*=0.072; denoted by #) was observed, with no effect of diet (*P*=0.27) or immobilization x diet interaction (*P*=0.61). * denotes post-hoc differences within a specific time point when comparing pre vs post immobilization.

Figure 5. Arterialised venous plasma L-[*ring*-$^{2}H_{5}$]phenylalanine (**A** and **B**) and [1-^{13}C]leucine (**C** and D) enrichments (Mole Percent Excess; MPE) pre- and post- one week of forearm immobilization in healthy young men fed a eucaloric control diet (A and C; CON; *n*=11) or a high-fat overfeeding diet (B and D; HFD; *n*=9) providing 50% excess energy from fat at 'background' (i.e. prior to intravenous tracer infusion; -20 min), in the postabsorptive state (0 min) and for a 3 h postprandial period following the ingestion of a mixed meal. CON and HFD data were compared within the same statistical test (3-way ANOVA with immobilization, time and diet as factors) for each labelled amino acid separately. For L-[*ring*-$^{2}H_{5}$]phenylalanine, a significant main effect of time (i.e. response to mixed meal ingestion; *P*<0.001) was observed, with no effect of diet (*P*=0.27), immobilization (i.e. pre vs post; *P*=0.61) or any interactions (all *P*>0.05). For [1-^{13}C]leucine, a significant main effect of time (*P*<0.001) was observed, with no effect of diet (*P*=0.177), immobilization (*P*=0.79) or any interactions (all *P*>0.05).

Figure 6. Forearm phenylalanine (**A** and **B**) and leucine (**C** and D) rate of disappearance pre- and post- one week of forearm immobilization in healthy young men fed a eucaloric control diet (A and C; CON; *n*=11) or a high-fat overfeeding diet (B and D; HFD; *n*=9) providing 50% excess energy from fat in the postabsorptive state (0 min) and for a 3 h postprandial period following the ingestion of a mixed meal. Inset figures represent the postprandial area under the curves (AUC). CON and HFD data were compared within the same statistical test (3-way ANOVA with immobilization, time and diet as factors) for each amino acid separately, with AUC data analysed with 2-way ANOVAs. For forearm phenylalanine rate of disappearance a significant effect of diet ($P<0.05$) was detected, with no effect of immobilization (i.e. pre vs post; $P=0.37$) or time (i.e. response to mixed meal ingestion; $P=0.11$), or any interactions evident (all $P>0.05$). For forearm phenylalanine rate of disappearance AUC, a significant effect of diet ($P<0.05$), but not immobilization ($P=0.26$) or immobilization x diet interaction ($P=0.26$) was observed. For forearm leucine rate of disappearance a significant effect of time ($P<0.05$), but no effect of immobilization ($P=0.50$) or diet ($P=0.13$) was observed. Significant immobilization x time ($P<0.05$) and immobilization x time x diet ($P<0.05$) interactions were detected, with other interactions not significant (both $P>0.05$). The 3-way interaction manifested as immobilization x time interactions in both the CON ($P<0.05$) and HFD ($P<0.05$), and time effects in the CON ($P<0.05$) and HFD ($P<0.05$) pre-, but not post- (both $P>0.05$, immobilization. For forearm leucine rate of disappearance AUC no significant effects were detected (all $P>0.05$). * denotes post-hoc differences within a specific time point when comparing pre vs post immobilization.

Figure 7. Forearm phenylalanine rate of appearance pre- and post- one week of forearm immobilization in healthy young men fed a eucaloric control diet (A; CON; *n*=11) or a high-fat overfeeding diet (B; HFD; *n*=9) providing 50% excess energy from fat in the postabsorptive state (0 min) and for a 3 h postprandial period following the ingestion of a mixed meal. Inset figures represent the postprandial area under the curves (AUC). CON and HFD data were compared within the same statistical test (3-way ANOVA with immobilization, time and diet as factors), with AUC data analysed with a 2-way ANOVA. No main effects of time (i.e. response to mixed meal ingestion; *P*=0.122), immobilization (i.e. pre vs post; *P*=0.784) or diet (*P*=0.19), or any interactions (all *P*>0.05) were detected, nor when any significant effects evident when analysing AUC data (all *P*>0.05).

Figure 1

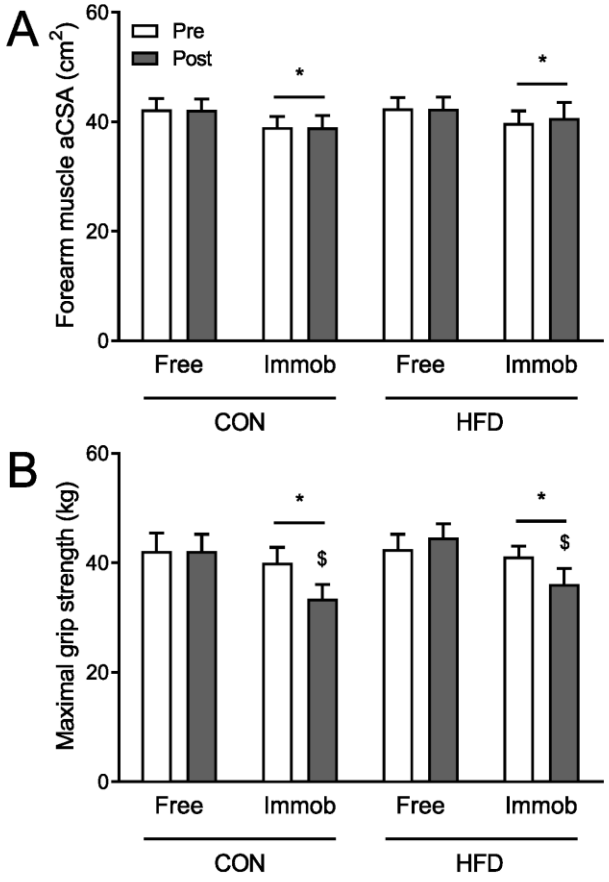

Figure 2

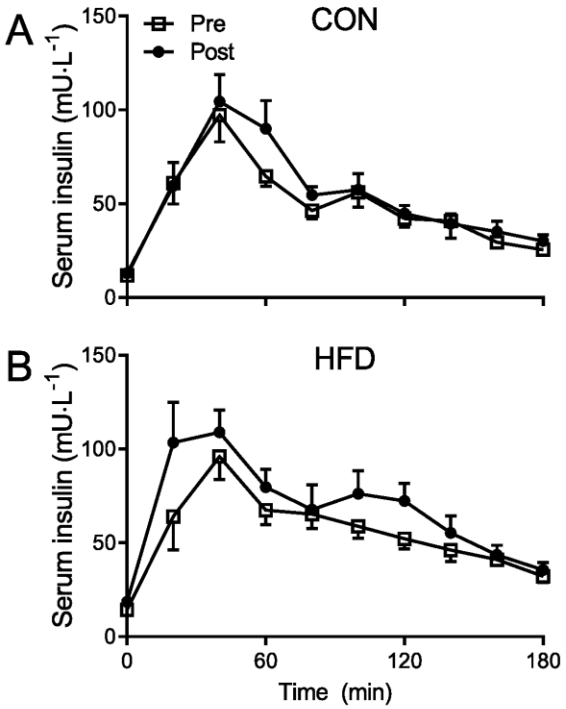

Figure 3

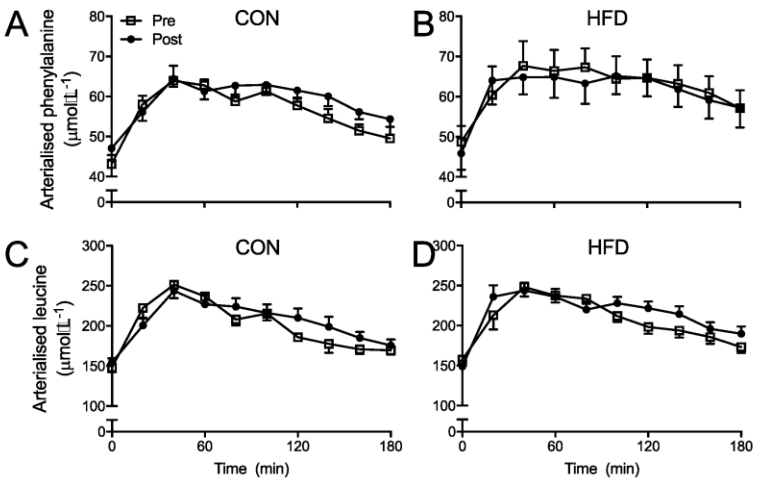

Figure 4

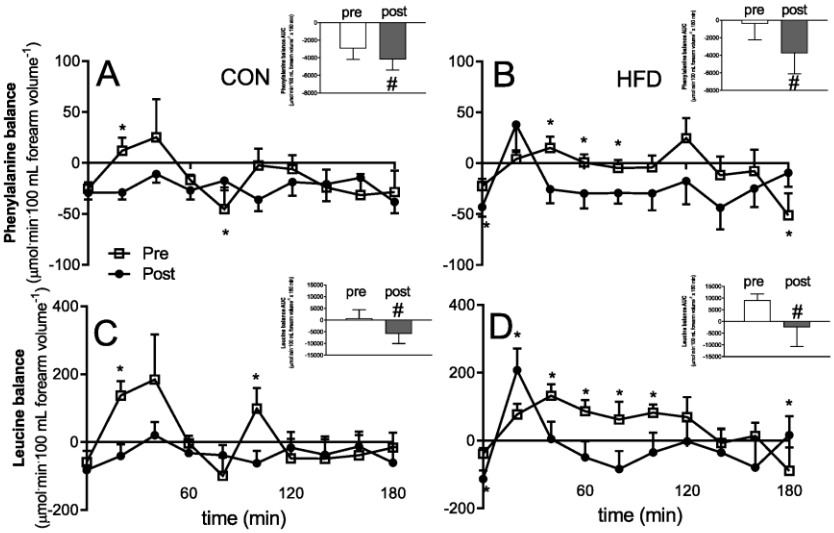

Figure 5

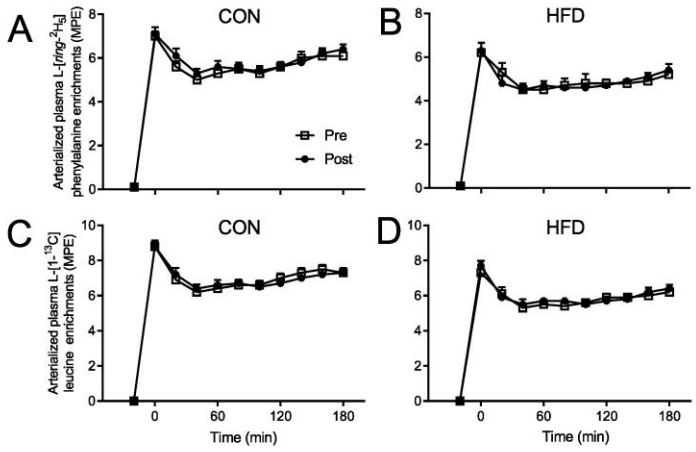

Figure 6

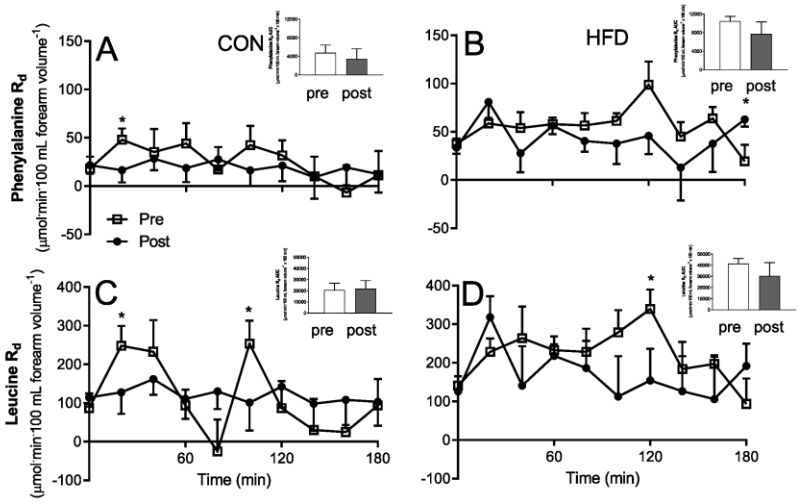

Figure 7