High-fat overfeeding does not exacerbate rapid changes in forearm glucose and fatty acid balance during immobilization

Marlou L. Dirks, Benjamin T. Wall, Britt Otten, Ana M. Cruz, Mandy V. Dunlop, Alan R. Barker, and Francis B. Stephens

Department of Sport and Health Sciences, College of Life and Environmental Sciences, University of Exeter, United Kingdom

Corresponding author:
Francis B. Stephens, 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
United Kingdom
Tel: +44 (0)1392 722157
Email: f.b.stephens@exeter.ac.uk

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Abstract

Context: Physical inactivity and high-fat overfeeding have been shown to independently induce insulin resistance. Objective: Establish the contribution of muscle disuse and lipid availability to the development of inactivity-induced insulin resistance. Design, setting, participants, and interventions: Twenty healthy males underwent seven days of forearm cast immobilization combined with a fully-controlled eucaloric (CON, n=10, age 23±2 yr, BMI 23.8±1.0 kg·m^{-2}) or high-fat diet providing 50% excess energy from fat (HFD, n=10, age 23±2 yr, BMI 22.4±0.8 kg·m^{-2}). Main outcome measures: Prior to casting, and following 2 and 7 days of immobilization, forearm glucose uptake (FGU) and non-esterified fatty acid (NEFA) balance were assessed using the arterialized venous-deep venous (AV-V) forearm balance method following ingestion of a mixed macronutrient drink. Results: Seven days of HFD increased body weight by 0.9±0.2 kg (P=0.002), but did not alter fasting, arterialized whole-blood glucose and serum insulin concentrations or the associated HOMA-IR or Matsuda indices. Two and seven days of forearm immobilization led to a 40±7% and 52±7% decrease in FGU, respectively (P<0.001), with no difference between day 2 and 7 and no effect of HFD. Forearm NEFA balance tended to increase following two and seven days of immobilization (P=0.095). Conclusions: forearm immobilization leads to a rapid and substantial decrease in FGU, which is accompanied by an increase in forearm NEFA balance but is not exacerbated by excess dietary fat intake. Altogether, our data suggest that disuse-induced insulin resistance of glucose metabolism ~~is~~ occurs as a physiological adaptation in response to the removal of muscle contraction.

Précis

Seven days of forearm immobilization leads to rapid changes in forearm glucose uptake and fatty acid balance. These changes are not exacerbated by ingestion of a high-fat, hypercaloric diet.

Introduction

Physical inactivity is a significant predictor of major non-communicable metabolic diseases including type 2 diabetes and cardiovascular disease (1), and has been proposed the 4th leading cause of death worldwide (2). A key hallmark of the physical inactivity-induced reduction in metabolic health is the development of insulin resistance of glucose metabolism, i.e. impaired glucose uptake in an insulin-stimulated state (1). Insulin resistance can also be induced via the removal of muscle contraction in an experimental setting, e.g. by subjecting individuals to acute muscle disuse via limb immobilization or bed rest (3). Although the development of insulin resistance with muscle disuse occurs much more rapidly than with chronic physical inactivity (i.e. a sedentary lifestyle), these experimental models represent suitable approaches to study physical inactivity in a more mechanistic manner.

It is well-established that experimental muscle disuse leads to insulin resistance of glucose metabolism (4-9), demonstrated by a 30-40% decrease in whole-body glucose uptake under hyperinsulinaemic-euglycaemic conditions following 7-9 days of bed rest, that plateaus for several weeks thereafter (10,11). We have recently shown that merely a single day of bed rest begins to reduce glucose tolerance and insulin sensitivity (12), which becomes frank insulin resistance following 3 days of bed rest (5,13,14). Taken together, this would suggest that a plateau in muscle insulin resistance may be reached within the first few days of disuse. However, bed rest may not be the most suitable experimental model to mechanistically study muscle disuse, since it is often (15,16), but not always (5,8,17), accompanied by a whole-body positive energy balance (15,16) and perturbed metabolism of other tissues (4,5). Limb immobilization on the other hand isolates muscle disuse whilst the rest of the body remains relatively active and in energy balance. Previous work has demonstrated that limb immobilization leads to a ~15-25% decrease in glucose uptake across the leg, as a direct measure of insulin sensitivity, following 7 days of disuse (9). The degree of insulin resistance with such 'isolated' disuse appears to be greater than with whole-body bed rest (6), but neither the time course nor the rapidity of its development have been established. Knowledge on the time course will provide insight in the underlying mechanism(s) of insulin resistance, i.e. establishing the order of occurrence of certain events can inform on potential causality. This is also of clinical relevance, since it will inform on when potential interventional

strategies (e.g. nutrition and/or exercise (mimetics) (18)) during periods of hospital admission or limb immobilization, are likely most effective.

One of the most commonly suggested mechanisms for the development of insulin resistance with muscle disuse is the reduced turnover and subsequent accumulation of lipid, or lipid intermediates, in skeletal muscle tissue (19). In non-disuse situations, lipid infusion directly impairs insulin sensitivity (20,21), and muscle lipid accumulation is strongly negatively correlated with insulin resistance (22). In line, prolonged (>4 weeks) bed rest and lower limb suspension studies have shown substantial skeletal muscle lipid accumulation, ranging from 15 to 75% increases in muscle lipid content following four weeks of muscle disuse (23,24). However, we have recently shown that such overt muscle lipid accumulation does not yet occur following 5-7 days of disuse (5,25), suggesting that the intramuscular accumulation of triglycerides *per se* is not involved in the rapid development of insulin resistance and might simply occur as a consequence. However, we have observed suggestions for specific changes in intramuscular diacylglycerol (DAG) metabolism in the first week of muscle disuse (5,12). Specifically, this was indicated by a trend for an increase in PKCθ gene expression following a single day of bed rest (12), potentially as a consequence of DAG-induced PKCθ activation, which is thought to impair muscle insulin signalling (19), and two-fold increases in muscle content of several different DAG species following 7 days of bed rest (5). These changes in DAG metabolism were likely caused by a reduced turnover (possibly due to a reduced demand for lipid as a fuel in inactive muscle tissue) or an excess muscle lipid uptake caused by positive energy balance. In our single-day bed rest study we tested the impact of 33% overfeeding during muscle disuse, and showed a negligible impact on measures of insulin sensitivity or muscle transcriptional responses (12). However, we overfed all macronutrients to a relatively moderate extent, which precluded us from delineating the role of excess lipid during disuse, and suggests a greater degree of specific high-fat overfeeding is perhaps required. As such, a greater degree of specific high-fat overfeeding (i.e., 50% excess energy, as in (26,27)) would potentially drive NEFA uptake in immobilized tissue, and thereby provide further mechanistic insight in the role of lipid in disuse-induced insulin resistance. Moreover, the results from this study have implications for population health, since physical inactivity and intake of a high-fat, high-caloric diet are becoming

increasingly prevalent in modern society and are key factors in non-communicable diseases such as type 2 diabetes and obesity.

In the present study we aimed to determine the effect of lipid availability on the development of inactivity-induced insulin resistance by applying the arterialized venous-deep venous (AV-V) forearm balance method prior to, and following 2 and 7 days of forearm immobilization in healthy young males consuming either a hypercaloric high-fat diet (i.e. 50% excess energy from fat) or eucaloric control diet. Forearm immobilization was selected as experimental model, since it induces muscle disuse in an isolated limb whilst only moderately impacting on activities of daily living (especially when compared to leg immobilization). In combination with the AV-V forearm balance technique, which is a feasible and relatively non-invasive method, this allowed us to assess the impact of muscle disuse on muscle substrate balance. The specific 2 and 7 day time points were selected to investigate whether the substantial insulin resistance that is observed following 7 days of muscle disuse can already be detected after 2 days, and assess whether this is associated with changes in forearm fatty acid balance. We hypothesized that a ~2.5-fold increase in dietary fat intake would create a positive lipid balance in the immobilized forearm tissue and therefore exacerbate the early development of insulin resistance, reflected by reduced forearm glucose uptake in response to a mixed macronutrient meal.

Methods

Participants

Twenty-two healthy, young men (age 23±1 y) were included in the present study. However, due to problems with cannulation and/or blood sampling on day 2 (*n*=1, control (CON) participant) and day 7 (*n*=1, high-fat diet (HFD) participant), data presented are for *n*=20. Participants' characteristics are displayed in **Table 1**. Prior to inclusion, participants attended the laboratory for a routine medical screening to ensure their eligibility to take part. 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 informed on the nature and risks of the experiment before written informed consent was obtained. After obtaining written informed consent, height and weight were measured, and body composition was determined by Air Displacement Plethysmography (Bodpod; Life Measurement, Inc., Concord, CA, USA). The present study was part of a larger project investigating the impact of forearm immobilization and high-fat overfeeding on muscle metabolism, registered on clinicaltrials.gov as NCT02980952. The study was approved by the Department of Sport and Health Sciences, University of Exeter's Ethical Committee (proposal reference number 161026/B/09) in accordance with the Declaration of Helsinki (version October 2013).

Experimental overview

Following inclusion, participants visited the laboratory for a baseline metabolic test day during which postabsorptive and postprandial forearm glucose uptake (FGU) and forearm non-esterified fatty acid (NEFA) balances were assessed using the arterialized venous-deep venous (AV-V) forearm balance method. Minimally 3 days later (average 10±2 days), participants attended the laboratory for the application of a forearm cast. This visit signified the beginning of the 7-day immobilization period.

During these 7 days, participants were randomized into receiving a fully controlled eucaloric (CON, *n*=10) or a high-fat (HFD, *n*=10) diet providing 50% excess energy from fat. Following 2 and 7 days of immobilization, the metabolic test day was repeated. The forearm cast was removed following the final test day.

Forearm immobilization

On the morning of the start of the 7-day immobilization period, participants arrived at the laboratory at 07:30 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 (Benecast™, BeneCare Medical, Manchester, UK) cast 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 all waking hours to keep the hand elevated above the elbow. A waterproof cover was provided to keep the cast dry whilst showering.

Dietary intake

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 intakes were calculated from these food diaries using online licensed software (28).

During the seven days of forearm immobilization, participants received a fully controlled diet, which was weighed out and prepared in a metabolic kitchen, from the research team. Participants received all individually packaged food products from the research team, and received instructions on how to cook the different meals via step-by step recipes. All meals and snacks were provided, whereas water and non-caloric drinks were allowed ad libitum. Energy requirements were calculated as basal metabolic rate (Henry equations, (29)) multiplied by an activity factor (International Physical Activity Questionnaire, IPAQ; (30)). Participants in the CON group received an individually tailored energy-balanced diet, containing 1.2 g protein·kg body weight^{-1}·d^{-1}. The target macronutrient composition was 50-55 energy percent (en%) carbohydrate, 30-35 en% fat, 10-15 en% protein, and 2 en% dietary fibre. Participants in the HFD group received a high-fat diet providing 50% excess energy from fat, which

was composed of the CON diet plus extra food products such as double cream and nuts, as well as larger amounts of cooking oils and sauces. Protein intake was identical to the CON group, at 1.2 g protein·kg body weight^{-1}·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 communication with the participants.

Experimental test day

Participants arrived at the laboratory at 08:00 in an overnight fasted state for the experimental test day (**Figure 1**). Body weight was measured with a digital balance with an accuracy of 0.1 kg (Seca, Hamburg, Germany). Participants rested on the bed in a semi-supine position for the entire experimental test day. Prior to the start of the experiment, cannulas were placed 1) retrograde into a dorsal hand vein of the non-immobilized hand for arterialized venous blood sampling, and 2) retrograde into a deep-lying antecubital vein of the (to-be) immobilized arm to sample venous blood draining the forearm muscle bed (31,32). The cannulated hand (with cannula 1)) was placed in a heated hand warmer (55°C). At *t*=0 min, 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 (7.2 kcal/kg body weight). This results in participants ingesting on average 71±2 g carbohydrates, 22±1 g protein, and 17±1 g fat. Arterialized venous and deep-venous blood was sampled simultaneously prior to drink ingestion (i.e. *t*=-20 and *t*=0), as well as every 20 min during the 3 hour postprandial phase. Prior to every blood sample, brachial artery blood flow of the (to-be) immobilized arm was determined by high-resolution ultrasound imaging in duplex mode (~12 MHz, Apogee, 1000. SIUI, China). Luminal diameter was imaged 5 cm proximal to the antecubital fossa for a 2 sec 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 (33). Files were analysed semi-automatically using Brachial Analyzer for Research, version 6.8.5 (Medical Imaging Applications LLC, Coralville, IA, USA, (34)). Forearm glucose uptake and forearm NEFA balance were calculated as the arterialized venous-deep venous

difference (AV-V) in glucose and NEFA concentrations, respectively, multiplied by brachial artery blood flow (35).

Sample analyses

Arterialized venous and deep-venous blood samples were collected for determination of whole-blood glucose and serum insulin and non-esterified fatty acid (NEFA) concentrations, and plasma cholesterol profile. Therefore, one part of every sample (0.5 mL) was collected in a BD Vacutainer® fluoride/oxalate tube, rolled on a tube roller for 2 min to inhibit glycolysis, and subsequently analysed for whole blood glucose concentrations (YSI 2300 PLUS, Yellow Springs, OH, USA). A second part (2 mL) was collected in BD Vacutainer® SST II tubes, which were left to clot at room temperature for ≥30 min and then centrifuged at 2,900*g* at 4°C for 10 min to obtain serum samples. Arterialized serum samples were used to determine insulin concentrations (Human insulin ELISA kit, DX-EIA-2935; Oxford Biosystems Ltd, Milton Park, UK). Arterialized venous and deep-venous serum samples were used for the determination of serum NEFA concentrations (Randox Laboratories Ltd, Crumlin, UK). Arterialized glucose and insulin concentrations were used to calculate the HOMA-IR (homeostatic model assessment of insulin resistance; (36)) and Matsuda (37) indices. The latter index was adjusted to capture the full 3 hour postprandial period, and calculated using the formula: Matsuda index = 10,000/√([fasting glucose x fasting insulin] x [mean glucose x mean insulin during 3 h postprandial period]). A third part of every sample (4 mL) was collected in BD Vacutainer® PST Lithium Heparin tubes and immediately centrifuged at 2,900*g* at 4°C for 10 min to obtain plasma samples. Fasting plasma triglyceride and cholesterol concentrations were measured in venous plasma samples using colorimetric assays on a Cobas 8000 modular analyser with 702 spectrophotometric module (Roche Diagnostics, Indianapolis, IN, USA).

Statistics

All data are expressed as means±SEM. Baseline characteristics between groups were tested using an independent samples *t*-test. Data were analysed using a Repeated Measures ANOVA with day (baseline vs day 2 vs day 7) and time (within the experimental test day) as within-subjects factor, and diet (CON

vs HFD) as between-subjects factor. In case of a significant interaction, Bonferroni post hoc tests were applied to locate individual differences. Pearson correlation coefficient was used to test for significant correlation between baseline indices for whole-body insulin sensitivity (i.e. HOMA-IR, Matsuda) and the change in FGU over 7 days of forearm immobilization. Statistical data analysis was performed using SPSS version 25.0 (IBM Corp, Armonk, NY, USA). Statistical significance was set at $P<0.05$.

Results

Dietary intervention

The two experimental groups did not differ in any of the participants' characteristics prior to the start of the study (**Table 1**). Habitual dietary intake was not different between the CON and HFD groups (**Table 2**; all variables between *P*=0.101 and *P*=0.987). The controlled diet during immobilization had a lower protein content (in g·d^{-1} and en%, *P*=0,003 and *P*=0.000, respectively) and higher carbohydrate content (in g·d^{-1}, *P*=0.000) than the habitual diet in both groups. During immobilization, the HFD group had a greater energy intake than the CON group (*P*=0.000), which was attributed to a ~2.5-fold greater fat intake (in g·d^{-1} and en%, both *P*=0.000). Saturated fat intake was 33±1 vs 126±5 g per day in the CON vs HFD groups, respectively (*P*=0.000). The controlled diet during immobilization resulted in a significant day*diet interaction for body weight (*P*=0.001), such that no change was observed in the CON group (from 75.0±4.2 to 74.5±4.2 to 74.3±4.1 kg on day 0, 2, and 7, respectively; *P*=0.103) but a significant increase was seen in the HFD group (from 71.9±3.1 to 72.0±3.1 to 72.8±3.1 kg on day 0, 2, and 7, respectively; *P*=0.002).

Whole-body glucose, insulin and NEFA concentrations, and calculated insulin sensitivity

Fasting arterialized blood glucose concentrations showed a tendency for a day*diet interaction (*P*=0.061), although no change was seen in the CON group (from 4.39±0.06 to 4.23±0.14 to 4.34±0.07 at baseline, day 2, and day 7, respectively; *P*=0.287) or HFD group (from 4.41±0.08 to 4.52±0.09 to 4.47±0.10 at baseline, day 2, and day 7, respectively; *P*=0.121). Instead, this trend was due to a tendency for a higher fasting glucose in the HFD on day 2 (*P*=0.096).

Arterialized fasting insulin concentrations showed a significant day effect (*P*=0.014), which was caused by increased insulin concentrations on day 2 and 7 (*P*=0.050 and *P*=0.040, respectively) in both groups, but no interaction or diet effect (interaction *P*=0.573, diet *P*=0.076). Specifically, fasting serum insulin concentrations in the CON group were 10.6±1.0, 13.0±2.0, and 12.7±1.4 mU·L^{-1} at baseline, day 2, and day 7, respectively, whereas serum insulin concentrations in the HFD group were 13.7±1.6, 16.4±1.6, and 17.6±1.8 mU·L^{-1}.

Arterialized fasting non-esterified fatty acid (NEFA) concentrations decreased by 32±12 and 21±16% on day 2 and day 7 (both *P*=0.000), respectively, with no difference between day 2 and 7 (*P*=0.959). Despite a visual difference (**Figure 2E** and **F**), no significant difference between groups was observed (*P*=0.122).

Arterialized blood glucose concentrations in response to mixed meal ingestion are displayed in **Figure 2A** and **2B**. Aside from a ~2.5 mmol·L^{-1} increase in arterialized glucose concentration following drink ingestion (time effect, *P*=0.000) a significant three-way interaction was observed (*P*=0.121), which was caused by a time*diet interaction on day 7 (*P*=0.004). This implies that after 7 days of forearm immobilization, postprandial glucose concentrations in the HFD at *t*=60, 80, and 100 min had returned back to fasting values (*P*-values between 0.096 and 0.942), whereas at those time points they were still elevated in the CON group (*P*-values between 0.000 and 0.009). Taken together, the 3 h postprandial area under the curve (AUC) for arterialized glucose concentrations (insets in **2A** and **2B**) was not different between diets or days (*P*=0.848 and *P*=0.323, respectively), indicating that the same amount of glucose was available in the total postprandial period.

Arterialized serum insulin concentrations in response to mixed meal ingestion are displayed in **Figure 2C** and **2D**. Serum insulin concentrations increased to 102.2±5.9 mU·L^{-1} after drink ingestion (time effect *P*=0.000), and trends for a day effect (*P*=0.066) and day*time*diet interaction (*P*=0.063) were observed. Despite these significant effects, the total 3 h postprandial AUC for serum insulin concentrations (insets in **2C** and **2D**) was not affected by immobilization or diet (*P*=0.080 and *P*=0.452, respectively).

Arterialized non-esterified fatty acid (NEFA) concentrations (**Figure 2E** and **F**) decreased substantially in the first hour after mixed meal ingestion (time effect, *P*=0.000), and showed a significant day effect and day*time interaction (*P*=0.005 and *P*=0.000, respectively). As a result, the total 3-h postprandial NEFA availability (insets) was 22±10% lower following 7 days of standardized nutrition (*P*=0.006). Despite a large visual difference in AUC between diets, this interaction effect only reached a trend for statistical significance (*P*=0.071), which was due to an effect of immobilization in HFD (*P*=0.001) but not in CON (*P*=0.718).

The HOMA-IR and Matsuda indices for insulin sensitivity are displayed in **Figure 3**. Although HOMA-IR was higher in HFD than in CON at all times (diet effect, *P*=0.049), CON and HFD showed similar ~15-30% increases with immobilization (day effect, *P*=0.014). The Matsuda index, a proxy for peripheral insulin sensitivity, tended to be lower at all times in HFD (*P*=0.052). Moreover, an 11±5% decrease in the Matsuda index was observed in both groups during the 7-day immobilization period (day effect, *P*=0.048). Neither HOMA-IR (Pearson's *r*=0.078, *P*=0.743) nor the Matsuda index (Pearson's *r*=0.147, *P*=0.536) at baseline correlated with the change in FGU over 7 days of immobilization, suggesting that baseline insulin sensitivity did not affect the response to immobilization with or without overfeeding.

Forearm glucose uptake

The arterialized venous to deep-venous (AV-V) forearm glucose difference demonstrated significant effects of immobilization and drink ingestion (both *P*=0.000), as well as an interaction between the two (*P*=0.000; data not shown). This interaction was caused by the AV-V glucose difference returning to fasting values after 140 min (baseline test day), 80 min (day 2), and 60 min (day 7), indicating that the postprandial difference in arterialised and venous glucose concentrations became smaller with immobilization. Brachial artery blood flow (**Figure 4A** and **B**) increased by 75±11% following drink ingestion (*P*=0.000), with no differences between groups or days (effect of diet *P*=0.898, interaction effect-*P*=0.478). The increase in blood flow was significantly different from fasting values from *t*=100 onwards on all test days (*P*-values between 0.001 and 0.047).

Figure 5 depicts forearm glucose uptake (FGU), calculated as the product of the AV-V glucose difference and brachial artery blood flow. Due to the lack of change in blood flow by immobilization or diet, FGU (**5A** and **B**) closely resembled the AV-V glucose difference. As such, significant effects of immobilization and drink ingestion were observed (both *P*=0.000), as well as a day*time interaction (*P*=0.002). This interaction was caused by FGU being different from fasting values between 20-120 min (baseline test day), 20-60 as well as 100-120 min (day 2), and 20 min (day 7; *P*-values between 0.000 and 0.047), indicating that the postprandial period of increased FGU decreased with immobilization. Total postprandial FGU (**Figure 5C** and **D**), calculated as the AUC over the 3 h

postprandial period, demonstrated a significant decrease with immobilization (day effect, *P*=0.001). More specifically, this was due to day 2 (-40±7%) and day 7 (-52±7%) being significantly lower than total FGU at baseline (*P*=0.002 and *P*=0.001, respectively), with no difference between day 2 and day 7 (*P*=1.000). No diet or interaction effect was observed (*P*=0.975 and *P*=0.995, respectively).

When FGU was expressed relative to arterialized glucose concentrations (i.e. as fractional glucose uptake) or relative to arterialized serum insulin concentrations, no difference between groups was observed (effects of diet: *P*=0.840 and *P*=0.962; day*diet interactions: *P*=0.931 and *P*=0.650, respectively).

Forearm NEFA balance

The AV-V forearm NEFA difference merely demonstrated a significant effect of drink ingestion (*P*=0.000; data not shown). The forearm NEFA balance, calculated as the product of the AV-V NEFA difference and brachial artery blood flow (**Figure 4)**, is depicted in **Figure 6A** and **B**. Similarly to the AV-V NEFA difference, an effect of feeding (*P*=0.008) but no other significant effects or interactions were observed. Despite a large visual increase in 3-h postprandial forearm net NEFA balance following 2 and 7 days of immobilization (**Figure 6C** and **D**), this only reached a tendency for a significant effect (*P*=0.095).

Circulating triglycerides and cholesterol

Fasting plasma triglyceride and cholesterol concentrations are depicted in **Figure 7**. Immobilization and HFD did not lead to changes in plasma triglyceride (**A**), total cholesterol (**B**), LDL cholesterol (**D**), or non-HDL cholesterol (**E**) concentrations (*P*-values for interaction effects between 0.112 and 0.300). A significant day*treatment interaction was observed for plasma HDL (**C**, *P*=0.019), such that an 18±4% increase in plasma HDL concentrations was observed after 7 days of the HFD (*P*=0.010). However, this did not result in changes in the cholesterol:HDL ratio (**F**, interaction effect *P*=0.186).

Discussion

The present study demonstrates that the profound insulin resistance observed with skeletal muscle disuse is already present after merely 2 days of forearm immobilization. This manifested as a ~50% reduction in forearm glucose uptake in response to a physiologically relevant mixed meal, and did not progress further in the subsequent 5 days. Insulin resistance was accompanied by a shift towards more positive net forearm NEFA balance, suggesting larger uptake and/or reduced release of NEFAs in forearm tissues. However, consumption of a high-fat, hypercaloric diet during immobilization did not exacerbate the development of insulin resistance, suggesting that inactivity induced insulin resistance is likely predominantly due to the lack of muscle contraction *per se*.

It is well-established that ≥1 week of muscle disuse leads to a substantial decrease in insulin-stimulated glucose uptake (4-9,11,14). Work by ourselves (5,12) and others (13) suggests that this insulin resistance develops between day 1 and 3 of muscle disuse, and plateaus off thereafter. Here we demonstrate for the first time that seven days of forearm immobilization leads to ~50% decrease in postprandial forearm glucose uptake (**Figure 5A** and **C**), measured via the arterialized venous-deep venous (AV-V) forearm balance method (38,39) in response to mixed meal ingestion. These data corroborate the aforementioned clamp studies showing a 30-40% decrease in insulin sensitivity following one week of disuse (4-8). Moreover, due to the use of the forearm balance method we now know that forearm immobilization did not affect the postprandial-mediated increase in brachial artery blood flow ((40); **Figure 4A**). We quantified that total postprandial forearm glucose uptake was 1.2 g during the total 3 h postprandial period on the baseline test day, which equates to 1.7% (i.e. 1.2 g of 71.3±2.4 g carbohydrates in the test drink) of ingested carbohydrates being taken up by the forearm muscles prior to immobilization. Assuming that forearm muscle mass accounts for 2% of whole-body muscle mass (i.e. 0.6 kg forearm muscle mass and 30 kg whole-body muscle mass, (32,38,41)) and glucose uptake is equal across all muscles, the 1.7% of glucose uptake taking place in the forearm muscles is directly in line with clamp data showing approximately 85% of glucose uptake takes place in muscle tissue (42). Importantly, this expands our knowledge by confirming earlier suggestions that bed rest-induced whole-body insulin resistance occurs primarily in skeletal muscle tissue (7), and supports the validity of the forearm balance technique to quantify glucose uptake as a direct measure of

muscle insulin sensitivity. Crucially, our novel data clearly demonstrate that the dramatic decline in insulin sensitivity occurs following merely two days of immobilization (**Figure 5**). Since the decrease in forearm glucose uptake was not linear but instead plateaued after two days (**Figure 5C**), our data suggest that the removal of muscle contraction *per se*, rather than duration of the disuse period, primarily dictates the degree of disuse-induced insulin resistance. Moreover, this plateau suggests that there is a certain basal level of glucose uptake that is reached when muscle contraction is taken away, and which cannot be reduced any further. The reason why this takes >1 day to occur (12) potentially has to do with protecting the muscle against daily periods of severely reduced muscle contraction, such as those occurring the overnight period. Physical inactivity of more than the normal daily ~8 hours of sleep might therefore be recognised as abnormal, and lead to the negative consequences observed here. Importantly, our data clearly demonstrate that in order to gain more insight into the mechanisms underlying disuse-induced insulin resistance, it is crucial to study muscle tissue within the first few days of muscle disuse.

The most commonly suggested mechanism for the development of insulin resistance with muscle disuse is the intramuscular accumulation of triglyceride (3,23,24) and/or lipid intermediates (5,12). To test the involvement of lipid in the development of immobilization-induced insulin resistance, we measured net forearm non-esterified fatty acid (NEFA) balance prior to, and following two and seven days of forearm immobilization. Here we demonstrate for the first time that net postprandial NEFA forearm balance tends to increase following merely two days of forearm immobilization (**Figure 6C**), accompanying the observed reduction in postprandial glucose uptake. This shift towards more positive net NEFA forearm balance can be caused by an increased muscle or adipose uptake, reduced adipose release, or a combination of these. Under normal non-disuse conditions, meal ingestion leads to a shift from adipose tissue fatty acid release (-1000 nmol·100 g adipose tissue^{-1}·min^{-1}) to fatty acid uptake (500 nmol·100 g adipose tissue^{-1}·min^{-1}), thereby creating a positive net fatty acid balance in the period 1 to 5 hours after meal ingestion (43). Simultaneously, muscle NEFA uptake decreases from ~115 at fasting levels to nearly 0 nmol·100 ml forearm volume^{-1}·min^{-1} with mixed meal ingestion (44), which is directly in line with our data (**Figure 6A**). Since the contribution of adipose tissue to forearm NEFA balance is several fold greater than the contribution of muscle, adipose tissue is therefore likely to contribute to changes

in forearm NEFA balance. Indeed, a reduction in whole-body and adipose tissue lipolysis has been shown in previous bed rest studies under basal and insulin-stimulated conditions (4,45), although the underlying mechanism remains to be established. However, since the temporal response of forearm NEFA balance became less negative with immobilization (**Figure 6A**) and it is unlikely that adipose tissue had become more insulin sensitive (i.e. taking up more NEFA in response to meal ingestion), it is possible that muscle tissue contributes to the more positive balance seen with disuse. A possible explanation for the observed shift towards more positive NEFA balance, which previous longer-term immobilization studies have failed to show (9,46), might be our novel approach of providing a mixed meal in the present study. This suggests that exogenous dietary fat plays an important role in the shift towards positive forearm NEFA balance, which is in line with previous work demonstrating that muscle disuse alters dietary fat trafficking (47). We have repeatedly shown that muscle triglyceride accumulation does not take place in the first 5-7 days of muscle disuse (5,25) but instead takes multiple weeks to occur (23,24), which is when insulin resistance has already plateaued off. This suggests that muscle triglyceride accumulation occurs in response to the development of insulin resistance, possibly as a consequence of a reduction in muscle fat oxidation (3,5) and likely influenced by a reduction in energy expenditure with disuse (5). However, the observed positive NEFA balance in this study might be related to the accumulation of specific lipid species such as diacylglycerols (DAGs), for which we have seen clear suggestions in our earlier work (5,12). As such, this study provides evidence for a potential role of lipid in disuse-induced insulin resistance, although future detailed mechanistic studies in the first few days of disuse are required to further elucidate this relationship.

In order to provide additional insight to the involvement of lipid in the development of disuse-induced insulin resistance, we aimed to drive muscle NEFA uptake via high-fat overfeeding. Specifically, participants were provided with a fully-controlled high-fat diet containing 50% excess energy, which increased total fat intake from ~100 g per day (in the control group) to ~270 g per day (in the high-fat overfed group, **Table 2**). Despite this substantial increase in dietary fat intake the high-fat overfed group showed a similar decline in forearm glucose uptake as the group fed in energy balance (**Figure 5**). Although contrary to our hypothesis, this shows that intake of excess lipid and energy via a high-fat, hypercaloric diet does not impact on insulin sensitivity of immobilized muscle tissue. Moreover, such

an increased dietary lipid intake did not affect forearm NEFA balance (**Figure 6B** and **D**). The lack of additional effect of the high-fat diet on forearm insulin sensitivity and NEFA balance are in line with the modest systemic changes caused by the high-fat diet. For example, high-fat overfeeding led to small increases in fasting glucose, but not fasting insulin, concentrations. Despite this, HOMA-IR increased with high-fat feeding to the same extent as the control group (**Figure 3A**). Moreover, the lack of impact on the modified Matsuda index suggests that high-fat overfeeding did not affect peripheral insulin sensitivity either (37), corroborating our forearm glucose uptake data. Despite this we are confident that participants adhered well to the provided diet. The ~1 kg gain in body mass was in line with other studies (26,27), as was the trend for a reduction in circulating fasting NEFA (**Figure 2F**; (48-50)) and increase in HDL cholesterol (**Figure 7C**). These changes are in line with previous work (26,49,50), and indicative of a substantial change in fatty acid composition of the diet (49). Although most studies show that short-term (i.e. 3-7 days) high-fat overfeeding induces insulin resistance (26,27,50-52), well-controlled studies showing no effect of high-fat feeding on insulin sensitivity also exist (48,49). The reason for the lack of impact on whole-body insulin sensitivity in our study is possibly the contribution of dietary fat to total energy in our high-fat diet (i.e. 55 en% fat, **Table 2**), which was somewhat lower than the 55-78 en% (average >65 en%) fat diets used in other studies (26,27,51,52). Indeed, our previous work demonstrated that even in isocaloric conditions, a 75% fat diet increases plasma FFA concentrations, inhibits pyruvate dehydrogenase complex (PDC), and reduces carbohydrate oxidation, which contribute to insulin resistance (53). This is supported by work from Lundsgaard and co-workers, who demonstrated that short-term overfeeding of dietary lipid, but not carbohydrate, decreases whole-body and leg glucose disposal under insulin clamp conditions in moderately trained individuals (52). However, since our diet was designed to contain the same absolute amount of carbohydrates and protein as the control diet (plus the addition of 50% extra energy from fat), increasing the relative fat content would have required a relative decrease in the other macronutrients, which is known to influence muscle metabolism during disuse (54). Altogether, despite the large amount of surplus fat ingested as part of the high-fat diet, our data demonstrate that this did not impact on systemic or local insulin sensitivity, or NEFA balance, in response to a physiologically relevant mixed meal, and therefore cannot provide further insight to the role of lipid accumulation in inactivity-induced insulin resistance.

The forearm balance technique is a well-established method to quantify forearm glucose uptake (9,11,14,39) and forearm NEFA balance (55,56), but to this point had not been applied to quantify forearm immobilization-induced insulin resistance and NEFA balance in the first days of muscle disuse. The simultaneous sampling of arterialized venous and deep-venous blood, which is directly draining forearm muscle tissue (39), represents a feasible and minimally invasive technique to study muscle metabolism *in vivo*. Moreover, due to the use of a mixed meal, the physiological response to meal ingestion can be quantified both systemically and locally, whilst the contribution of circulating glucose and insulin concentrations can be corrected for. Moreover, the method could be applied to study the balance of other nutrients (e.g. amino acid metabolism) or nutrient subspecies. However, an important limitation of this two-pool model is the lack of muscle tissue sampling, which precludes us from taking measurements of intracellular signalling/processes/fuel stores to gain further insight into the mechanisms underlying insulin resistance and a shift towards positive NEFA balance. We speculate that a likely mechanism is the removal of muscle contraction leading to a lack of stimulus for GLUT4 translocation to the plasma membrane. Indeed, although muscle GLUT4 translocation following disuse has never been measured, bed rest has been shown to decrease muscle GLUT4 protein expression (17,57). Future work should aim to elucidate the role of GLUT4 translocation in the development of insulin resistance in the first few days of muscle disuse.

A first limitation to the study we would like to acknowledge is that despite thorough dietary control, based on well-established methods (29,30), the control group demonstrated a non-significant decline in body weight. Despite this, we are confident that our approach in overfeeding lipid was successful as it resulted in significant body mass gains in the high-fat overfed group. Secondly, although the decrease in whole-body insulin sensitivity might be a consequence of ingesting a controlled diet instead of a habitual diet, a reduction in physical activity during the immobilization period may also contribute. Although we consider it unlikely that forearm immobilization decreases physical activity to this extent, we unfortunately did not collect physical activity data to support this. Thirdly, the used test meal was moderately high in carbohydrates and fat (i.e. containing 71±2 and 17±1 g carbohydrates and fat, respectively), and it may have impacted on our results if a meal of different composition was used. Indeed, an extensive meta-analysis has previously shown that dietary macronutrient composition of a

test meal directly effects postprandial insulin sensitivity (58). Lastly, although we clearly demonstrate that the disuse-induced development of insulin resistance is accompanied by an increase in forearm NEFA balance, future work adopting a stable isotope fatty acid tracer approach is required to determine the fate and origin of the NEFAs.

We conclude that short-term forearm cast immobilization leads to the rapid development of severe peripheral insulin resistance. Crucially, this develops entirely in the first two days of immobilization, suggesting a physiological adaptation to reduced substrate requirements rather than a pathological condition. Insulin resistance occurs in parallel with a shift towards more positive forearm NEFA balance, which might in part be explained by an increased muscle NEFA uptake but is more likely due to decreased adipose tissue lipolysis. Although the provision of excess lipids via high-fat overfeeding did not exacerbate insulin resistance, the shift towards more positive NEFA balance means we cannot rule out a contribution of lipid to inactivity-induced insulin resistance. Irrespective of this, our data demonstrating the rapid decrease and subsequent plateau in insulin sensitivity suggest that the removal of muscle contraction is a key contributor to disuse-induced insulin resistance. As such, this stresses the relevance of incorporating some level of muscle contraction immediately following the onset of muscle disuse, in order to maintain metabolic health.

Competing interests

None of the authors disclose any conflicts of interest.

Author contributions

MLD, BTW and FBS designed the study. MLD, BTW, BO, AMC, MVD, ARB, and FBS organised and carried out the clinical experiments. MLD, BO, and AMC carried out the laboratory analyses. MLD performed the (statistical) analyses. MLD, BTW, and FBS interpreted the primary data. MLD drafted, and BTW and FBS edited and revised the manuscript. All authors approved the final version.

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Figure legends

Figure 1: Outline of the experimental test days, performed at baseline, and after 2 and 7 days of forearm immobilization in *n*=20 healthy, young males.

Figure 2: Arterialized venous glucose (**A+B**) and insulin (**C+D**) concentrations at baseline and following 2 and 7 days of forearm immobilization in healthy young males fed a eucaloric control diet (CON; **A+C**) or a high-fat diet providing 50% excess energy from fat (HFD; **B+D**). The insets represent the total area under the curve (AUC) for the 180 min postprandial period following ingestion of a mixed meal drink. For arterialized venous glucose concentrations, a time*treatment interaction was observed for day 7 (P<0.01). On that day, postprandial glucose concentrations in the HFD at t=60, 80, and 100 min were no longer different from fasting values (all P>0.05), whereas they were still elevated in the CON group (all P<0.05). For arterialized venous insulin concentrations, a time effect was observed (P<0.05). Arterialized venous non-esterified fatty acid (NEFA) concentrations are displayed for participants in the CON (**E**) and HFD (**F**) groups. Significant day, time, and day*time effects were observed (all P<0.05). * Significantly different from baseline.

Figure 3: Indices of whole-body insulin sensitivity at baseline and following 2 and 7 days of forearm immobilization in healthy young males fed a eucaloric control diet (CON) or a high-fat diet providing 50% excess energy from fat (HFD). Open bars: baseline; grey bars: day 2, black bars: day 7. A: Homeostatic Model Assessment of Insulin Resistance (HOMA-IR). B: Matsuda index. * Significantly different from Baseline (P<0.05). $ Significantly different from CON (P=0.049). # Trend for a difference between CON and HFD (P=0.052).

Figure 4: Brachial artery blood flow (measured via Doppler Ultrasound) following consumption of a mixed meal drink (at t=0) prior to, and following 2 and 7 days of forearm immobilization in healthy young males. During immobilization, participants consumed a eucaloric control diet (CON; **A**) or a diet

containing 50% excess energy from fat (HFD; **B**). Blood flow was significantly higher than fasting values from *t*=100 onwards on every test day (*P*<0.05).

Figure 5: Forearm glucose uptake (FGU) at baseline and following 2 and 7 days of forearm immobilization in healthy young males fed a eucaloric control diet (CON, **A**) or a high-fat diet providing 50% excess energy from fat (HFD, **B**). A significant day*time interaction (*P*<0.01) was observed, due to FGU being different from fasting values between 20-120 min (Baseline test day), 20-60 as well as 100-120 min (Day 2), and 20 min (Day 7; all *P*<0.05). Panels **C** (CON) and **D** (HFD) represent total forearm glucose uptake, calculated as area under the curve (AUC) from **A** and **B**, over the entire 3 hour postprandial period. * Significantly different from baseline test day (*P*<0.05).

Figure 6: Forearm non-esterified fatty acid (NEFA) balance at baseline and following 2 and 7 days of forearm immobilization in healthy young males fed a eucaloric control diet (CON, **A**) or a high-fat diet providing 50% excess energy from fat (HFD, **B**). A significant effect of feeding was observed (*P*=0.000), independent of diet (interaction effect *P*=0.927). Panels **C** (CON) and **D** (HFD) represent total forearm NEFA balance, calculated as area under the curve (AUC) from **A** and **B**, over the entire 3 hour postprandial period. The increase in NEFA AUC following 2 and 7 days of immobilization tended to be statistically significant (*P*=0.095). # Trend for an effect of immobilization (*P*=0.095).

Figure 7: Fasting plasma triglyceride and cholesterol concentrations in healthy young males at baseline and after 2 and 7 days of a eucaloric control diet (CON, *n*=10) or high-fat diet providing 50% excess energy from fat (HFD, *n*=10). * Significantly different from baseline (*P*=0.004) and day 2 (*P*=0.029).

Table 1: Participants´ characteristics

	CON (*n*=10)	HFD (*n*=10)
Age (y)	23 ± 2	23 ± 2
Body mass (kg)	75.1 ± 4.1	71.9 ± 3.0
Height (m)	1.77 ± 0.02	1.79 ± 0.02
BMI (kg·m^{-2})	23.8 ± 1.0	22.4 ± 0.8
Body fat (%)	14.3 ± 3.7	11.7 ± 2.6

Values represent means ± SEM. BMI, body mass index. No significant differences were observed between groups.

Table 2: Dietary intake

	CON (*n*=10)		HFD (*n*=10)	
	Habitual	**Immobilization**	**Habitual**	**Immobilization**
Energy (MJ·d^{-1})	11.5 ± 1.0	11.9 ± 0.3	10.8 ± 0.8	18.2 ± 0.6 * #
Protein (g·kg BW^{-1}·d^{-1})	1.70 ± 0.17	1.21 ± 0.00 *	1.67 ± 0.18	1.20 ± 0.00 *
Protein (g·d^{-1})	126 ± 14	91 ± 5 *	119 ± 12	86 ± 3 *
Carbohydrates (g·d^{-1})	311 ± 24	370 ± 8 *	265 ± 18	381 ± 12 *
Fat (g·d^{-1})	103 ± 13	105 ± 3	111 ± 12	267 ± 8 * #
Fibres (g·d^{-1})	29 ± 4	30 ± 1	22 ± 1	36 ± 1 * #
Protein (En%)	19 ± 1	13 ± 1 *	18 ± 1	8 ± 0 * #
Carbohydrate (En%)	46 ± 1	52 ± 1 *	42 ± 2	35 ± 0 * #
Fat (En%)	33 ± 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 was calculated and provided by the research team. During immobilization, participant were fed a fully-controlled eucaloric diet (CON) or a high-fat diet providing 50% excess energy from fat (HFD). Abbreviations: BW, body weight; 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).