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ARTICLE

Bed rest and resistive vibration exercise unveil novel links between skeletal muscle mitochondrial function and insulin resistance

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Abstract

Aims/hypothesis Physical inactivity has broad implications for human disease including insulin resistance, sarcopenia and obesity. The present study tested the hypothesis that (1) impaired mitochondrial respiration is linked with blunted insulin sensitivity and loss of muscle mass in healthy young men, and (2) resistive vibration exercise (RVE) would mitigate the negative metabolic effects of bed rest.

Methods Participants (n = 9) were maintained in energy balance during 21 days of bed rest with RVE and without (CON) in a crossover study. Mitochondrial respiration was determined by high-resolution respirometry in permeabilised fibre bundles from biopsies of the vastus lateralis. A hyperinsulinaemic–euglycaemic clamp was used to determine insulin sensitivity, and body composition was assessed by dual-energy x-ray absorptiometry (DEXA).

Results Body mass (−3.2 ± 0.5 kg vs −2.8 ± 0.4 kg for CON and RVE, respectively, $p < 0.05$), fat-free mass (−2.9 ± 0.5 kg vs −2.7 ± 0.5 kg, $p < 0.05$) and peak oxygen consumption ($\dot{V}O_{2peak}$) (10–15%, $p < 0.05$) were all reduced following bed rest. Bed rest decreased insulin sensitivity in the CON group (0.04 ± 0.002 mg kgFFM^{-1} [pmol l^{-1}] min^{-1} vs 0.03 ± 0.002 mg kgFFM^{-1} [pmol l^{-1}] min^{-1} for baseline vs post-CON), while RVE mitigated this response (0.04 ± 0.003 mg kgFFM^{-1} [pmol l^{-1}] min^{-1}). Mitochondrial respiration (oxidative phosphorylation and electron transport system capacity) decreased in the CON group but not in the RVE group when expressed relative to tissue weight but not when normalised for citrate synthase activity. LEAK respiration, indicating a decrease in mitochondrial uncoupling, was the only component to remain significantly lower in the CON group after normalisation for citrate synthase. This was accompanied by a significant decrease in adenine nucleotide translocase protein content.

Conclusions/interpretation Reductions in muscle mitochondrial respiration occur concomitantly with insulin resistance and loss of muscle mass during bed rest and may play a role in the adaptations to physical inactivity. Significantly, we show that RVE is an effective strategy to partially prevent some of the deleterious metabolic effects of bed rest.

Keywords Bed rest · Energy expenditure · Exercise · Insulin resistance · Metabolism · Mitochondrial function · Skeletal muscle

Abbreviations

ANT	Adenine nucleotide translocase
CHO	Carbohydrate
CON	Control

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ETS	Electron transport system
FA	Fatty acid
OXPHOS	Oxidative phosphorylation
RMR	Resting metabolic rate
RVE	Resistive vibration exercise
SUIT	Substrate-uncoupler-inhibitor titration
TMPD	*N*,*N*,*N*′,*N*′-Tetramethyl-p-phenylenediamine dihydrochloride
UCP3	Uncoupling protein 3
$\dot{V}O_{2peak}$	Peak oxygen consumption

Introduction

The long-term consequences of prolonged physical inactivity probably contribute to the progression of insulin resistance and the development of type 2 diabetes [1–3]. Bed rest is a unique model of physical inactivity where ambulatory movement is eliminated and energy balance can be rigorously controlled. Bed rest provides a physiological context to interrogate the relationships between changes in muscle mass [4], metabolic rate [5] and insulin sensitivity [6], and can provide valuable insights into the aetiology of type 2 diabetes.

There has been much focus on nutritional countermeasures to mitigate the negative effects of bed rest by inducing protein synthesis and preventing muscle loss through using amino acids alone [7] or combined with exercise [8] and whey protein [9, 10]. However, results to date are equivocal. Resistance exercise has shown potential by increasing muscle protein synthesis [11], insulin sensitivity [12], mitochondrial enzyme activity [13] and mitigating muscle mass loss during bed rest [14]. Vibration exercise, with high frequency, improves insulin sensitivity in animals [15] but has little effect on muscle atrophy during bed rest [16]. However, when combined, resistance and vibration exercise have shown additive benefits on muscle and bone during bed rest [17]. These data support resistive vibration exercise (RVE) as a novel therapeutic countermeasure that may also provide mechanistic insight into the relationship between mitochondrial function and insulin resistance.

When investigating the mechanisms underpinning insulin resistance and muscle loss induced by physical inactivity it is critical that energy balance is maintained. Otherwise, lower physical activity leads to a positive energy balance (as reviewed in Bergouignan et al [5]) that increases fat mass, decreases insulin sensitivity [18] and accelerates muscle loss [19]. When energy balance is maintained, muscle mass alone decreases [20, 21]. Positive energy balance and elevated fat mass can cause mitochondrial dysfunction in skeletal muscle, which confounds the study of bed rest-induced insulin resistance per se. However, a recent report indicated that skeletal muscle lipid content was not changed when energy balance and fat mass were preserved during a 7 day bed rest intervention, even though there was a significant decrease in insulin sensitivity [22]. If intramyocellular lipotoxicity is not mediating the changes in insulin sensitivity during bed rest per se, the implication is that alterations to mitochondrial function may be responsible.

Skeletal muscle mitochondria play a critical role in regulating cellular energy homeostasis and have been implicated in insulin resistance [23] and the regulation of muscle mass [24, 25]. It has been suggested that a decrease in mitochondrial lipid metabolism causes insulin resistance and it is a matter of debate if changes are due to dysfunctional mitochondria or a reduction in content [26–29]. While proteomic and transcriptomic analysis following bed rest demonstrate decreased expression of oxidative pathways and enzymes [22, 30], there is a need for more detailed investigations.

The purpose of this study was to determine the impact of 21 days of bed rest on mitochondrial respiration in skeletal muscle, insulin resistance and metabolic rate. We hypothesised that bed rest-induced physical inactivity would decrease mitochondrial respiration and that this reduction would be associated with decreased insulin sensitivity and energy expenditure. In addition, we hypothesised that these changes would be mitigated by a novel RVE intervention.

Methods

Participants A total of 12 healthy men volunteered to participate in a 21 day, three arm crossover design, bed rest study. This analysis was conducted on those participants who completed the control (CON) and RVE trials as there was insufficient mitochondrial respiration data from the third crossover arm. Of the 12 participants, mitochondrial respiration data for two individuals was not valid because of poor permeabilisation and one individual dropped out of the study after the first trial; therefore, the analysis was conducted on data from nine participants (Table 1). All participants provided written informed consent and underwent a comprehensive screening protocol. Ethical approval was obtained from the Comité de Protection des Personnes/CPP Sud–Ouest Outre-Mer I and the French

Table 1 Baseline characteristics

	CON (n = 9)	RVE (n = 9)
Age (years)	33.7 ± 2.4	33.7 ± 2.4
Height (m)	1.77 ± 0.02	1.77 ± 0.02
Weight (kg)	71.13 ± 2.9	70.96 ± 2.6
BMI (kg/m^2)	22.7 ± 0.9	22.6 ± 0.8
$\dot{V}O_{2peak}$ (ml/min)	2830 ± 165	2731 ± 176

Data are presented as mean ± SE

Health Authorities (Agence Française de Sécurité Sanitaire des Produits de Santé).

Study outline The experimental protocol was a randomly assigned crossover design where all volunteers completed 21 days of the CON and RVE trials, separated by a washout period of at least 4 months. The CON trial required volunteers to remain in a supine position with at least one shoulder on the bed at all times. The RVE trial was similar except volunteers performed five sessions of RVE on a specially designed ergometer, remaining supine at all times. Participants resided at the testing facility for 7 days before and after the bed rest period to standardise preparation and allow baseline and recovery data to be collected. As this study is part of a European Space Agency project, the volunteers were in a −6° head down tilt position for the duration of bed rest (i.e. the bed is tilted so that the person's head is slightly below the level of their feet) to mimic the vascular changes that are associated with microgravity [31].

Energy balance and body composition Body mass, body composition using dual-energy x-ray absorptiometry (DEXA) and resting metabolic rate (RMR) were measured throughout bed rest. Peak oxygen consumption ($\dot{V}O_{2peak}$) and maximal isometric voluntary contraction were measured using standard protocols before and after bed rest. (See electronic supplementary material [ESM] Methods Energy balance and body composition and Aerobic fitness and isometric strength.)

Hyperinsulinaemic–euglycaemic glucose clamp A two stage hyperinsulinaemic–euglycaemic glucose clamp was performed at baseline prior to the first period of bed rest and on day 19 of the CON and RVE trials. Insulin was i.v. infused using a precision pump at 0.25 μU kg^{-1} min^{-1} and 1 μU kg^{-1} min^{-1} along with low dose heparin (10,000 U/100 ml saline [0.9% NaCl]). Each stage was 240 min and blood glucose levels were maintained at 5 mmol/l using a 10% dextrose variable rate infusion that was adjusted every minute according to continuously measured blood glucose values determined in arterialised blood (heated-hand technique). The glucose infusion rate, used to estimate insulin sensitivity, was calculated over the last 60 min of the second stage and is expressed relative to the circulating insulin concentration (pmol/l) and fat-free mass (kgFFM).

RVE protocol The exercise training was performed on days 2, 5, 12, 16 and 20. All training was performed on an integrated training device (Novotec Medical, Pforzheim, Germany). This device combines a standard leg press machine, previously used for bed rest studies [31], with a vibration platform located on the foot plate. While the participants were completing the leg press protocol the foot plate was vibrating (8 mm peak-to-peak, 25 Hz). Participants performed bilateral squats (ten repetitions, 75% 1-RM, 8 s/rep), single heel raises (×1.3 body weight, contractions performed as fast as possible until fatigue) and bilateral heel raises (×1.8 body weight, contractions performed as fast as possible until fatigue). A 5% load adjustment was made based on the ability of volunteers to complete the set of exercises. The exercise protocol is provided in detail in ESM Table 1 and a photograph is provided in ESM Fig. 1.

Skeletal muscle analysis Skeletal muscle biopsies were used for protein quantification (ESM Table 2), muscle fibre analysis, citrate synthase activity and mitochondrial respiration. In brief, substrate-uncoupler-inhibitor titration (SUIT) protocols for carbohydrate (CHO) and fatty acid (FA) respiration were performed in saponin-permeabilised skeletal muscle fibres. LEAK and oxidative phosphorylation (OXPHOS) respiration, as well as Complex I and II linked electron transport system (ETS) capacity and Complex IV activity was determined with the addition of various substrates and inhibitors. (See ESM Methods Skeletal muscle biopsies and Mitochondrial respiration experimental protocols.)

Statistical analysis The data are expressed as mean ± SE. The Shapiro–Wilk test was used to determine if the data was normally distributed, and if not the data was log_{10}-transformed for analysis. A mixed between–within subjects analysis of variance was conducted to assess the impact of the two bed rest trials (CON and RVE) on the physiological variables (body mass, body composition, RMR, aerobic capacity and muscle strength). There were no significant differences in the baseline values prior to each trial indicating that there were no carry-over effects attributable to the crossover design. This was verified in a separate analysis using independent samples *t* tests for each of the physiological variables. The differences in insulin sensitivity between baseline and post-CON and post-RVE were determined using a one-way ANOVA. There were differences in the baseline values for a number of the skeletal muscle variables. Therefore, a within-group analysis was performed using a paired sample *t* test and between-group comparisons were not performed. A Pearson correlation coefficient was used to establish if there was a relationship between variables. The statistical analysis was performed in SPSS 22.0 considering a two-sided 0.05 significance level.

Results

Body composition Body mass decreased progressively during bed rest (Fig. 1) and there was a significant effect of time but not trial. The decrease in body mass could be attributed to a decrease in fat-free mass, which followed a similar pattern of

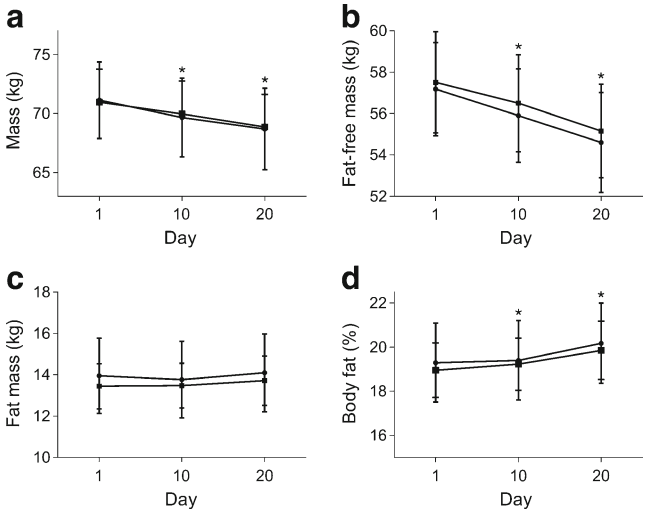

Fig. 1 Changes in body weight and body composition. Body mass (**a**), fat-free body mass (**b**), fat mass (**c**) and per cent body fat (**d**) during the CON (circles) and RVE (squares) trials. Data are presented as mean ± SE. *$p < 0.05$ vs day 1

decline. Fat mass was maintained during bed rest and there were no differences between or within trials (CON 14.0 ± 1.7 kg to 14.1 ± 1.7 kg; RVE 13.5 ± 1.0 kg to 13.7 ± 1.1 kg).

Physiological performance There was a significant effect of time, but not trial, on $\dot{V}O_{2peak}$ (Fig. 2a, b) when expressed in absolute terms (l/min) and relative to fat-free mass. Peak power was significantly reduced following bed rest (Fig. 2d; $p < 0.05$) but there were no differences in peak heart rate (Fig. 2c). Ankle flexion and extension, and knee extension but not flexion, were significantly decreased following bed rest (Fig. 2e–h; $p < 0.05$). When all values were normalised for fat-free mass (data not shown) there were no differences in strength measures, suggesting that the reduction in fat-free mass accounts for the decrease in strength and power.

Metabolic alterations Compared with baseline (0.04 ± 0.002 mg kgFFM^{-1} [pmol/l]$^{-1}$ min^{-1}), glucose infusion rate (Fig. 3a) decreased significantly following the CON (0.03 ± 0.002 mg kgFFM^{-1} [pmol/l]$^{-1}$ min^{-1}, $p < 0.05$) but not the RVE trial (0.037 ± 0.003 mg kgFFM^{-1} [pmol/l]$^{-1}$ min^{-1}). There was a significant effect of time on RMR (Fig. 3b) with a similar progressive decrease in both trials on days 5, 10, 15 and 21 ($p < 0.05$). However, substrate use, as measured by RQ, did not change during either trial (data not shown).

Mitochondrial respiration In both the CHO and FA SUIT experiments (Fig. 4a, b), O_2 flux during LEAK (state 4) respiration was significantly lower in the CON group (2.9 ± 0.5 to 1.2 ± 0.4 pmol [s × ml]$^{-1}$ mg^{-1}, $p < 0.05$) after 21 days of bed rest but not in the RVE group (2.1 ± 0.4 to 1.5 ± 0.3 pmol [s × ml]$^{-1}$ mg^{-1}). A similar pattern was evident with the addition of other substrates and inhibitors. After bed rest, ADP-stimulated state 3 respiration was significantly lower in the CON group (18.9 ± 2.0 to 12.0 ± 2.0 pmol [s × ml]$^{-1}$ mg^{-1}, $p < 0.05$), with preserved function in the RVE group (15.6 ± 2.9 to 15.6 ± 1.7 pmol [s × ml]$^{-1}$ mg^{-1}). Maximal respiration through Complex I of the electron transport chain, as determined with carbonyl cyanide-4-[trifluoromethoxy] phenylhydrazone (FCCP) titrations, and Complex I and II with the addition of succinate was also significantly lower for the CON ($p < 0.05$) but not the RVE trial. Subsequent inhibition of Complex I by rotenone blunted respiration during the CHO SUIT in the CON group only ($p < 0.05$). The addition of cytochrome *c* did not change oxygen flux. There were no differences in Complex IV activity as determined by the addition of ascorbate and the artificial electron donor TMPD (*N*,*N*,*N*′,*N*′-tetramethyl-p-phenylenediamine dihydrochloride).

To assess the intrinsic mitochondrial function, all oxygen fluxes were normalised to citrate synthase activity. We also normalised the data to the OXPHOS protein cocktail and the overall results were similar (data not shown). Citrate synthase activity (Table 2) was significantly decreased after both the CON and RVE trials ($p < 0.05$). After normalisation (Fig. 4c, d), there were no significant differences in the oxygen flux during the CON trial, with the exception of LEAK respiration. This difference was still evident (Fig. 5a) with subsequent normalisation of LEAK respiration by wet weight, Complex I protein content, and Complex I–Complex IV mitochondrial protein content ($p < 0.05$).

Respiratory ratios were calculated to assess the impact of bed rest on intrinsic respiratory performance (Fig. 6). In the CON trial, LEAK respiration normalised to ETS (LEAK/ETS) was significantly decreased in the CHO SUIT ($p < 0.05$), indicating a decrease in uncoupled respiration. The respiratory control ratio, which is the ADP-stimulated respiration relative to LEAK respiration (OXPHOS/LEAK), is an indicator of ADP-coupled respiration, often referred to as state 4/state 3. This ratio was increased ($p < 0.05$) during the CHO SUIT after the CON trial, indicating an increase in coupled respiration. There were no significant changes in respiratory ratios during the RVE trial.

Muscle fibre characteristics and mitochondrial protein quantification The muscle fibre composition and cross-sectional area was assessed in a subgroup of the participants ($n = 5$) because of insufficient biopsy specimens. There was no change in the muscle fibre type after bed rest (data not shown) but there was a significant decrease in the cross-sectional area of both fast and slow twitch muscle fibres (ESM Fig. 2). An OXPHOS cocktail of ETS proteins were measured using western blot. There was a trend towards a reduction in Complex III after the CON ($p = 0.08$) and RVE ($p = 0.09$) trials. No other changes were noted in mitochondrial ETS proteins (ESM Fig. 3). In order to investigate the mechanism that might contribute to the decrease in LEAK

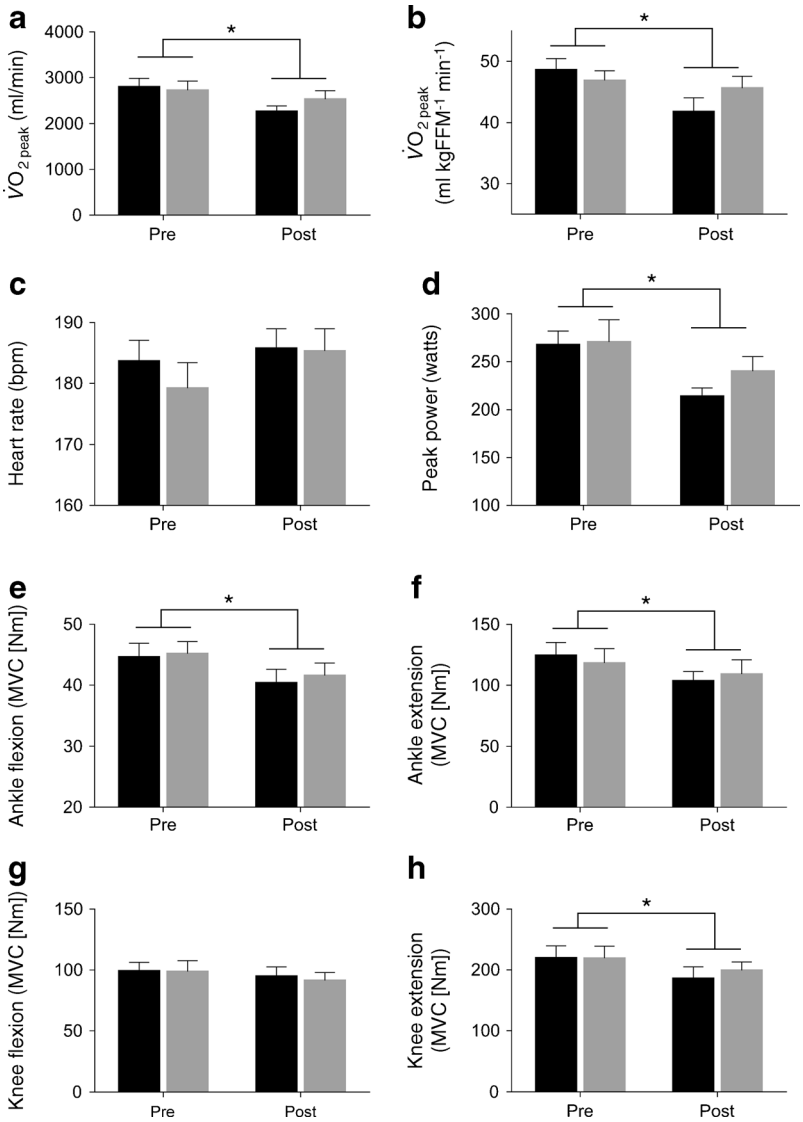

Fig. 2 Aerobic capacity and isokinetic strength before (pre) and after (post) 21 days of bed rest. $\dot{V}O_{2peak}$ is presented in absolute values (ml/min) (**a**) and relative to fat-free mass (ml kgFFM^{-1} min^{-1}) (**b**). Peak heart rate (**c**) and peak power (**d**) were also determined. Maximal isometric voluntary contraction (MVC) was determined using isokinetic strength measurements during ankle flexion (**e**) and extension (**f**) and knee flexion (**g**) and extension (**h**). Black bars, CON; grey bars, RVE. Data are presented as mean ± SE. *$p < 0.05$ vs before bed rest as an effect of time

respiration following the CON trial, even when normalised to markers of mitochondrial content, the protein levels of skeletal muscle uncoupling protein 3 (UCP3) and adenine nucleotide translocase (ANT) were determined (Fig. 5b, c). There was a significant reduction in ANT levels following bed rest in the CON trial while UCP3 levels were decreased in the RVE trial ($p < 0.05$).

Correlation analysis When we combined the data for before and after each trial (Table 3), there was a positive correlation between LEAK respiration in the CHO SUIT and $\dot{V}O_{2peak}$ ($r = 0.47$, $p < 0.01$), RMR ($r = 0.56$, $p < 0.01$) and insulin sensitivity ($r = 0.33$, $p < 0.05$). There was also a correlation for OXPHOS respiration with RMR ($r = 0.35$, $p < 0.05$), and for maximal ETS capacity with RMR ($r = 0.40$, $p < 0.01$) and insulin sensitivity ($r = 0.35$, $p < 0.05$). A similar relationship in the FA SUIT was noted for OXPHOS with RMR ($r = 0.37$, $p < 0.05$) and insulin sensitivity ($r = 0.44$, $p < 0.01$). Maximal ETS in the FA SUIT was also positively correlated with insulin sensitivity ($r = 0.35$, $p < 0.05$).

Discussion

Insulin resistance is a key adaptation to prolonged physical inactivity that probably contributes to loss of muscle mass and increased risk of type 2 diabetes. Despite its pathophysiological importance, there is much controversy surrounding the mechanisms underpinning insulin resistance and its translation to human disease or pathobiology. We show for the first time that 21 days of bed rest reduced skeletal muscle mitochondrial respiration concomitant with decreased insulin sensitivity, metabolic rate and fat-free mass. While there are many integrated cascades contributing to insulin resistance, this study suggests that a decrease in mitochondrial respiration may mediate some

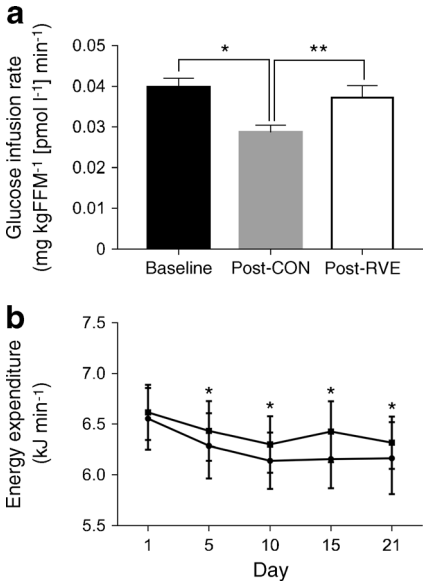

Fig. 3 Insulin sensitivity and the RMR following bed rest. Insulin sensitivity, as measured by glucose infusion rate, (**a**) was determined using a hyperinsulinaemic–euglycaemic clamp at baseline (black bar), post-CON (grey bar) and post-RVE (white bar). The glucose infusion rates are expressed relative to fat-free mass (kgFFM) and the circulating insulin concentration (pmol/l). The RMR (**b**) was measured by indirect calorimetry (kJ/min) at five time points throughout bed rest. Data are presented as mean ± SE. *$p < 0.05$, **$p < 0.001$

of the changes linked to physical inactivity. Importantly, many of the negative effects of bed rest were mitigated by completing five sessions of RVE. Given the increasing evidence that periods of immobilisation may contribute to the progression of type 2 diabetes, these data provide novel mechanistic insights into the detrimental metabolic and physiological effects of bed rest.

The decrease in insulin sensitivity following 21 days of bed rest was similar to previously reported bed rest studies [5]. This decrease was not evident following the RVE trial, indicating that the exercise protocol was sufficient to maintain insulin sensitivity. In addition, the correlation between insulin sensitivity and aerobic capacity supports the association between these variables in healthy young men [32]. We report an 8–10% decrease in RMR and a 10–15% reduction in $\dot{V}O_{2peak}$ following the CON and RVE trials, consistent with other studies [33, 34]. It is clinically important to note that the changes in insulin sensitivity occurred despite maintaining energy balance and support the importance of physical activity in any preventive strategy.

It is well established that mitochondrial content and enzyme activity increase in response to exercise training [35], independent of age [36] or type 2 diabetes [37]. However, the impact of physical inactivity on mitochondrial content or function has received little or no attention. In this study, we found a global decrease in skeletal muscle mitochondrial respiration following 21 days of bed rest. This is in contrast to a

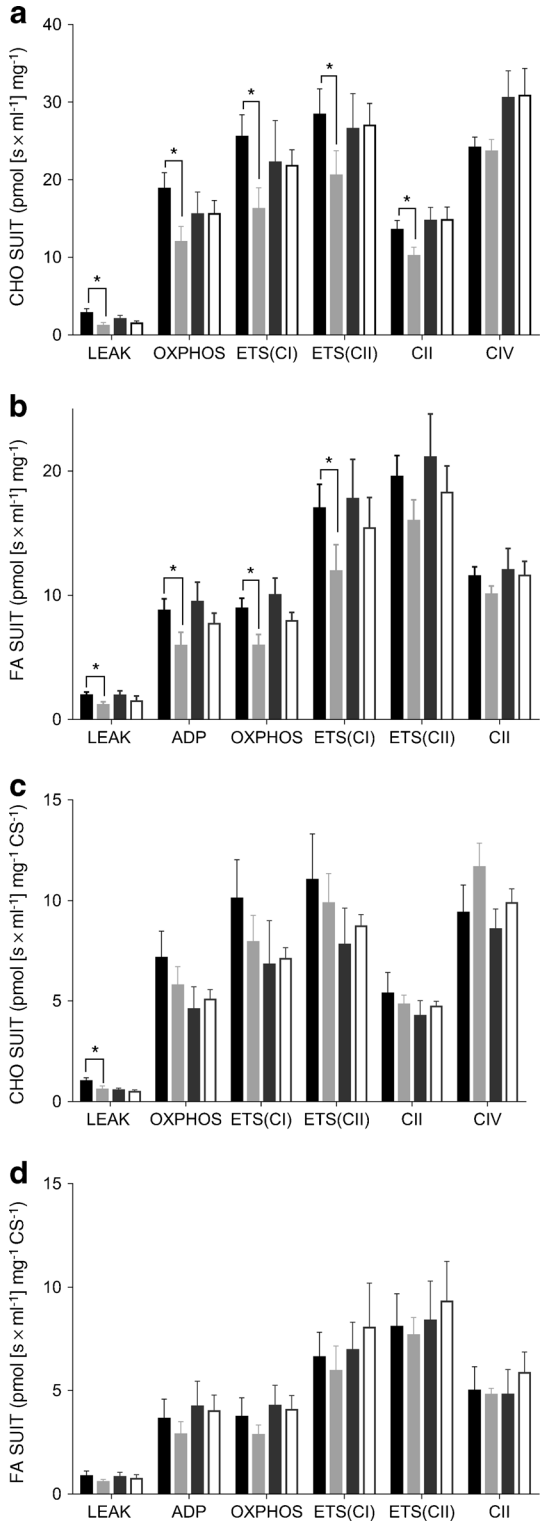

Fig. 4 Mitochondrial respiration data for the CHO SUIT (**a**) normalised to tissue wet weight and the FA SUIT (**b**) normalised for tissue weight. Both the CHO SUIT (**c**) and FA SUIT (**d**) data were also normalised to tissue wet weight and citrate synthase activity. Black bars, pre-CON; light grey bars, post-CON; dark grey bars, pre-RVE; white bars, post-RVE; ETS(CI), maximal respiration through Complex I; ETS(CII), maximal respiration through Complex I and Complex II; CII, respiratory capacity through Complex II only when Complex I is inhibited; CIV, Complex IV. Data are presented as mean ± SE. *$p < 0.05$

Table 2 Mitochondrial protein expression and activity

	CON		RVE	
	Baseline	Post-trial	Baseline	Post-trial
Citrate synthase activity (μmol ml^{-1} min^{-1})	3.03 ± 0.44	2.19 ± 0.22*	3.68 ± 0.31	3.12 ± 0.25*
OXPHOS cocktail				
Complex I	1.0 ± 0.2	1.0 ± 0.2	1.4 ± 0.3	1.8 ± 0.3
Complex II	1.7 ± 0.2	1.6 ± 0.1	1.7 ± 0.2	1.5 ± 0.1
Complex III	1.4 ± 0.2	1.2 ± 0.1‡	1.3 ± 0.1	1.2 ± 0.1†
Complex IV	1.3 ± 0.3	1.2 ± 0.2	1.0 ± 0.2	1.0 ± 0.1
Complex V	1.2 ± 0.2	1.1 ± 0.1	1.2 ± 0.2	1.0 ± 0.2
COXIV	0.9 ± 0.2	0.9 ± 0.2	1.2 ± 0.3	1.0 ± 0.2
SDHa	0.7 ± 0.1	0.8 ± 0.1	1.2 ± 0.2	1.3 ± 0.3

Values (mean ± SE) represent citrate synthase activity and protein quantification from western blot analysis normalised for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) before and after 21 days of bed rest for the CON and the RVE trials

*$p < 0.05$, † $p = 0.09$, ‡ $p = 0.08$ vs baseline

recent report following 10 days of bed rest where no differences were reported [38]. It may therefore take a period of time greater than 10 days for mitochondrial respiration to decrease. There is a need for careful time course studies of mitochondrial adaptations to bed rest to provide the next level of evidence. Importantly, the changes observed in our study following bed rest were not evident following the RVE protocol, indicating a protective role of RVE on mitochondrial respiration. These findings may have important implications for managing people during hospitalisation and support a role for physical activity in the recovery of metabolic health following periods of immobilisation. While preserved mitochondrial function is evident with RVE, these findings do not translate to whole body aerobic capacity. This discrepancy may be explained by the fact that $\dot{V}O_2$ is influenced by both central (pulmonary diffusing capacity, stroke volume, heart rate and systemic oxygen extraction) and peripheral factors (oxygen carrying capacity, partial pressure of oxygen, capillary density and mitochondrial enzyme level) [39]. While many variables can impact whole body aerobic capacity, the measurement of mitochondrial respiration in skeletal muscle permeabilised muscle fibres gives us a more direct and informative readout of the capacity of skeletal muscle to consume oxygen.

The changes observed in mitochondrial respiration were evident when expressed relative to tissue wet weight, but most were not present when data was expressed relative to citrate synthase activity. A similar pattern has been reported when mitochondrial respiration has been normalised to content following limb immobilisation [40], in older individuals [41] or in people with metabolic disease [29], indicating that the changes are possibly due to reduced mitochondrial content. In addition, the relative contribution of substrate use remained the same following bed rest and there were no differences in Complex IV activity, as measured by the addition of TMPD and ascorbate. Collectively, these data suggest that the decrease in mitochondrial respiration observed following bed rest was proportional to a reduction in mitochondrial density.

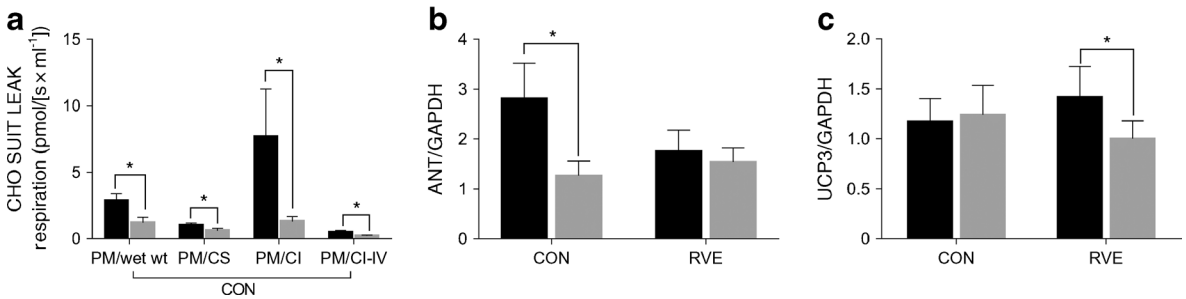

Fig. 5 LEAK respiration and mitochondrial uncoupling protein content. The LEAK (state 4) respiration (PM, pyruvate and malate) from the CHO SUIT (**a**) was normalised for wet weight, wet weight and citrate synthase (CS), Complex I protein content and total mitochondrial OXPHOS protein content (Complex I–Complex IV) pre (black bars) and post (grey bars) bed rest. The protein content for mitochondrial uncoupling proteins ANT (**b**) and UCP3 (**c**) were determined by western blot in the CON and RVE trials pre (black bars) and post (grey bars) bed rest. Representative blots are presented in ESM Fig. 4. Data are expressed as mean ± SE. *$p < 0.05$ vs pre-values as an effect of time

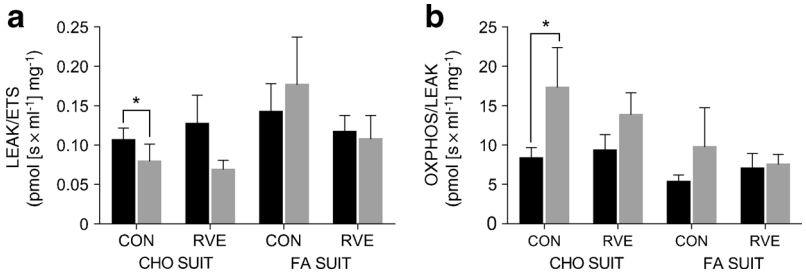

Fig. 6 Mitochondrial respiratory ratios. The LEAK control ratio (LEAK/ETS) (**a**) and the respiratory control ratio (OXPHOS/LEAK) (**b**) were determined for each trial before (black bars) and after (grey bars) bed rest in the CON and RVE trials. Data are expressed as mean ± SE. *$p < 0.05$ vs before bed rest as an effect of time

However, the investigation of mitochondrial function remains a challenge and other mechanisms cannot be ruled out.

One of the most interesting findings from this study was the significant decrease in LEAK respiration. LEAK respiration is an indicator of mitochondrial uncoupling and refers to the transport of protons from the inner mitochondrial space to the matrix without producing ATP [42]. Reduced LEAK persisted in the CON trial when the carbohydrate, but not lipid, substrate data was normalised for a number of mitochondrial markers, including citrate synthase activity and OXPHOS protein content. The carbohydrate LEAK respiration correlated with aerobic capacity, RMR and insulin sensitivity suggesting a partial contribution of decreased LEAK to these physiological outcomes. It has been reported that ~50% of respiration in skeletal muscle is proton leak and ~14% of the metabolic rate could be attributed to this process [43]. However, further research is needed to determine if the decrease in LEAK respiration is causative, or a consequence, of the decreased energy demand and metabolic rate. These associations were not evident for the FA SUIT and suggest that the regulation of carnitine palmitoyltransferase (CPT1) may be more important for determining the impact of lipids on mitochondrial respiration [42].

The LEAK control ratio (LEAK/ETS) normalises LEAK respiration to the ETS capacity and the ratio increases with uncoupling [42]. In the CHO SUIT, we found a decrease in LEAK/ETS indicating a decrease in mitochondrial uncoupling following bed rest in the CON group (Fig. 6a). On the other hand, an increase in the respiratory control ratio (OXPHOS/LEAK) was only found in the CON group (Fig. 6b). The respiratory control ratio is an indicator of ADP-coupled respiration, also referred to as state 3/state 4 [44]. Collectively, these ratios suggest that, in response to bed rest, the mitochondria favour coupled respiration instead of LEAK respiration and this may be a compensatory mechanism to maintain normal mitochondrial function. These data are supported by reports of decreased LEAK respiration following 2 weeks of limb immobilisation in young and elderly men [40]. These results may be significant when examining the impact of decreased physical activity on the process of ageing as LEAK respiration is significantly lower in elderly individuals [45], but there are no reported differences associated with type 2 diabetes [27, 29]. As there was no reduction in LEAK in the RVE trial, these data reinforce the importance of physical activity as a long-term decrease in LEAK respiration is associated with increased reactive oxygen species production and oxidative damage. While this study was conducted in young healthy adults, the results identify some of the early metabolic changes associated with physical inactivity but further studies will be required to demonstrate the long-term impact on the processes of ageing.

We wanted to determine if there was a cellular mechanism that could account for the decrease in LEAK respiration following bed rest; therefore, we measured the levels of uncoupling proteins. UCP3 is the most widely studied skeletal muscle uncoupling protein but we did not observe a change in UCP3 levels in the CON trial. Interestingly, we did find a decrease in UCP3 levels following the RVE trial. In skeletal muscle, UCP3 accounts for a small proportion of total LEAK respiration and UCP3 knockout mice have unchanged

Table 3 Correlation analysis for mitochondrial respiration and physiological variables

	RMR (*r*)	Insulin sensitivity (*r*)	CHO respiration (*r*)			FA respiration (*r*)		
			LEAK	OXPHOS	ETS	LEAK	OXPHOS	ETS
$\dot{V}O_{2peak}$	0.41**	0.63**	0.47**	0.26	0.21	0.04	0.25	0.17
RMR		0.40*	0.56**	0.40**	0.35*	0.15	0.37*	0.24
Insulin sensitivity			0.33*	0.32‡	0.35*	0.21	0.44**	0.35*

*$p < 0.05$, **$p < 0.01$, ‡$p = 0.06$

respiration rates and proton conductance [46] suggesting an alternative regulatory system. A second type of mitochondrial carrier that can cause inducible proton conductance, or uncoupling, is ANT. We found a significant decrease in ANT1/2 levels in the CON but not the RVE trial. In ANT1 knockout mice there is a significant decrease in state 4 respiration, accounted for by a 50% decrease in proton leak kinetics [47]. In addition, UCP3 knockout mice display increased proton conductance but no change in maximal LEAK respiration after undergoing 2 weeks of energy intake restriction [48]. The role of ANT in proton conductance is not fully understood but it does not depend on the function of ANT as a nucleotide translocase, FA compositional changes or the inner membrane bilayer surface area. It may depend on a change in the properties of proton conductance at the ANT and phospholipid interface or be indirectly correlated with the ATP demand of the cell [47]. Therefore, a change in the expression of ANT could be an important factor in regulating uncoupled respiration in human skeletal muscle and may explain the decrease in LEAK respiration observed following bed rest.

While studies such as this are highly controlled human experiments they have limitations that should be acknowledged. Given the resources required and the need for careful and detailed investigation of participant phenotype it is not possible to include a large number of individuals. In addition, this was a crossover study and while it allows for a comparison of the same participants, there is a risk of variability between trials. There were no differences in the baseline physiological variables suggesting the 4 month washout period was sufficient but there were differences in some of the skeletal muscle variables and we used within-group analysis; for these data. Finally, bed rest studies cause fluid shifts and increase diuresis and these changes may influence the fat-free mass measured by DEXA.

In conclusion, we provide evidence for reduced mitochondrial respiration after 21 days of bed rest. The reduction in cellular respiration was associated with decreased insulin sensitivity and metabolic rate and it is possible that these changes are inter-related in response to physical inactivity. The decline in mitochondrial respiration may be due to reduced mitochondrial content as evidenced by the decrease in citrate synthase activity; however, further studies are required and other mechanisms cannot be excluded. There were no declines in mitochondrial function observed after the RVE trial suggesting that the mode and frequency of exercise had a partially protective effect. After the CON trial, CHO SUIT LEAK respiration was reduced and this was consistently evident when normalised to other mitochondrial variables. The reduction in the LEAK control ratio and the increase in the respiratory control ratio suggest that the mitochondria are adapting to maintain normal function during bed rest.

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Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author contribution HK and DO'G had full access to all the data in the study and take full responsibility for the integrity of the data and the accuracy of the data analysis. The study was designed by HK, FR, DO'G, SB and MH. HK, FR, DO'G and SB performed all physiological measurements. HK, FR, DO'G, SB, LB and DB performed the muscle analyses. MS, DB and TH contributed to the analysis and interpretation of fibre typing. The data was acquired by HK, SB, DO'G, FR, MH, TH, MS and DB. The data was interpreted and discussed by all authors. HK and DO'G drafted the manuscript and all authors were involved in revising the article for important intellectual content. Approval of final manuscript was given by all authors.

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