

**Short-term bed rest-induced insulin resistance cannot be explained by increased
mitochondrial H₂O₂ emission**

Marlou L. Dirks¹, Paula M. Miotto², Gijs H. Goossens¹, Joan M. Senden¹, Heather L.
Petrick², Janneau van Kranenburg¹, Luc J.C. van Loon¹, and Graham P. Holloway²

¹*NUTRIM School of Nutrition and Translational Research in Metabolism, Maastricht
University Medical Centre⁺, the Netherlands*

²*Human Health & Nutritional Sciences, University of Guelph, Guelph, Ontario, Canada*

Address for correspondence:

Graham P. Holloway, PhD

University of Guelph

Department of Human Health and Nutritional Sciences

Guelph, On, Canada, N1G 2W1

Email: ghollowa@uoguelph.ca

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Abstract

Mitochondrial H₂O₂ has been causally linked to diet-induced insulin resistance, however it remains unclear if muscle disuse similarly increases mitochondrial H₂O₂. Therefore, we investigated the potential that an increase in skeletal muscle mitochondrial H₂O₂ emission, potentially as a result of decreased ADP sensitivity, contributes to cellular redox stress and the induction of insulin resistance during short-term bed rest in twenty healthy males. Bed rest led to a decline in glucose infusion rate during a hyperinsulinemic-euglycemic clamp ($-42\pm 2\%$; $P<0.001$), and in permeabilized skeletal muscle fibres decreased OXPHOS protein content ($-16\pm 8\%$) and mitochondrial respiration across a range of ADP concentrations ($-13\pm 5\%$). While bed rest tended to increase maximal mitochondrial H₂O₂ emission rates ($P=0.053$), H₂O₂ emission in the presence of ADP concentrations indicative of resting muscle, the ratio of H₂O₂ emission to J_{O₂} consumption, and markers of oxidative stress were not altered following bed rest. Altogether, while bed rest impairs mitochondrial ADP-stimulated respiration, an increase in mitochondrial H₂O₂ emission does not contribute to the induction of insulin resistance following short-term bed rest.

Keywords: mitochondrial respiration, H₂O₂ emission, ROS, insulin sensitivity, muscle disuse

Key Points

- We determined if bed rest increased mitochondrial derived reactive oxygen species and cellular redox stress, contributing to the induction insulin resistance
- Bed rest decreased maximal and submaximal ADP-stimulated mitochondrial respiration
- Bed rest did not alter mitochondrial H₂O₂ emission in the presence of ADP concentrations indicative of resting muscle, the ratio of H₂O₂ emission to JO₂ consumption, or markers of oxidative stress
- The present data strongly suggests that mitochondrial H₂O₂ does not contribute to bed rest-induced insulin resistance

Abbreviations

ADP, adenosine diphosphate; ANT, adenine nucleotide translocase; ATP, adenosine triphosphate; CS, citrate synthase; DAG, diacylglycerol; DXA, Dual Energy X-Ray Absorptiometry; EE, energy expenditure; FFM, fat-free mass; GIR, glucose infusion rate; GS, glycogen synthase; GSH, reduced glutathione; GSSH, oxidized glutathione; GSH:GSSG, ratio between reduced and oxidized glutathione; HbA1c, glycated haemoglobin; HK, hexokinase; IC₅₀, median inhibition concentration; JO₂, mitochondrial O₂ flux; Km, Michaelis constant; OXPHOS, oxidative phosphorylation; RCR, respiratory control ratio; RER, respiratory exchange ratio; ROS, reactive oxygen species; VDAC, voltage-dependent anion channel; 4HNE, 4-Hydroxynonenal

Introduction

Various situations of injury or illness require a period of muscle disuse to enhance recovery. Although such periods of bed rest or immobilization are necessary, they are associated with negative health consequences such as a decline in muscle mass (Deitrick, 1948; Ingemann-Hansen & Halkjaer-Kristensen, 1980; Dirks *et al.*, 2015), functional capacity (Deitrick, 1948; Ingemann-Hansen & Halkjaer-Kristensen, 1980), and basal metabolic rate (Tzankoff & Norris, 1977; Haruna *et al.*, 1994). Moreover, muscle disuse has been shown to rapidly lead to the development of whole-body insulin resistance, with a 20-30% decline in insulin sensitivity observed following 7-9 days of bed rest (Stuart *et al.*, 1988; Alibegovic *et al.*, 2009; Dirks *et al.*, 2016). Impairments in skeletal muscle insulin sensitivity are thought to contribute substantially to these responses (Stuart *et al.*, 1988), however, the underlying mechanism(s) remain to be established.

While there is a paucity of literature explaining the mechanisms causing short-term bed rest-induced insulin resistance, we have recently shown that intramuscular reactive lipid concentrations do not increase during bed rest (Dirks *et al.*, 2016). Alternatively, mitochondrial-derived reactive oxygen species (ROS) have been proposed to play a key role, as decreasing mitochondrial ROS emission (Anderson *et al.*, 2009; Lee *et al.*, 2010; Kang *et al.*, 2012; Sakellariou *et al.*, 2016) prevents diet-induced insulin resistance. In addition, attenuating mitochondrial ROS in rodents prevents disuse-mediated atrophy (Min *et al.*, 2011), further suggesting that mitochondrial-mediated redox signalling contributes substantially to the cellular changes induced by muscle disuse.

Biologically, ADP binding to F₁F₀ ATP synthase decreases membrane potential and the overall rate of superoxide production (Anderson *et al.*, 2007; Picard *et al.*, 2010). As such, approaches

that decrease the provision of ADP to mitochondria likely contribute to the development of insulin resistance. Importantly, a variety of experimental models have suggested that mitochondrial ADP sensitivity is impaired in situations where mitochondrial content is reduced (Dudley *et al.*, 1987; Phillips *et al.*, 1996). In support of this, bed rest has recently been shown to decrease the protein content of the inner mitochondrial membrane ADP transporter, adenine nucleotide translocase (ANT, (Kenny *et al.*, 2017)). Moreover, we have previously shown that the ability of submaximal ADP concentrations to decrease mitochondrial hydrogen peroxide emission is impaired in insulin resistant rodents (Smith *et al.*, 2013; Miotto *et al.*, 2018). Altogether, these data suggest that increased mitochondrial ROS emission, potentially as a result of impaired ADP sensitivity, contributes to the induction of insulin resistance. However, it remains to be determined if such an increase in mitochondrial ROS emission also underlies short-term bed rest-induced insulin resistance.

Therefore, we investigated the potential link between mitochondrial bioenergetics and disuse-induced insulin resistance in healthy young males undergoing one week of bed rest. Specifically, the primary aim of this study was to determine the impact of bed rest on mitochondrial ADP sensitivity, redox balance and mitochondrial H₂O₂ emission. We hypothesized that bed rest would decrease mitochondrial ADP sensitivity and, as a result, increase mitochondrial ROS emission in the presence of submaximal ADP concentrations and markers of *in vivo* oxidative stress. Additionally, we hypothesized that continuous food delivery, typically applied as tube feeding in clinical practice, would exaggerate the development of insulin resistance (Stoll *et al.*, 2012), potentially by increasing mitochondrial ROS. By combining muscle tissue analyses with repeated hyperinsulinemic-euglycemic clamps, we were able to assess the impact of changes in mitochondrial bioenergetics and dietary substrate delivery on disuse-induced insulin resistance.

Methods

Subjects

Twenty healthy, recreationally active males (age 25 ± 1 y) were included in the present study. Subjects' characteristics are presented in **Table 1**. Prior to inclusion, subjects completed a general health questionnaire and visited the University for a medical screening to ensure their eligibility to take part. Exclusion criteria were a BMI below 18.5 or above $30 \text{ kg} \cdot \text{m}^{-2}$, a (family) history of deep vein thrombosis, type 2 diabetes mellitus (determined by HbA1c values $>7.0\%$), and any back, knee or shoulder complaints that could be problematic during bed rest. Furthermore, subjects who had been involved in progressive resistance-type exercise training during the 6 months prior to the study were excluded. All subjects were informed on the nature and risks of the study prior to obtaining written informed consent. The current study was part of a larger project investigating the impact of short-term bed rest on skeletal muscle mass and function, registered on clinicaltrials.gov as NCT02521025 (Dirks *et al.*, 2019). Approval for the study was obtained from the Medical Ethical Committee of Maastricht University Medical Centre⁺ (registration number MEC 15-3-035), in accordance with the Declaration of Helsinki.

Experimental outline

One day prior to bed rest, subjects visited the university where (in the following order) a skeletal muscle biopsy was collected, and a hyperinsulinemic-euglycemic clamp and DXA scan were performed (test day 1). The following morning, participants started a 7-day period of strict bed rest. On the morning of day 8, after exactly 7 days bed rest, the test day was repeated (test day 2). During the 7 days prior to bed rest, as well as the entire 7-day bed rest period, food intake was fully controlled. Resting energy expenditure was determined immediately at the start and end of bed rest as previously reported (Omnical; Maastricht

University, Maastricht, the Netherlands, (Schoffelen *et al.*, 1997)). VO_2 peak tests were performed 2 days before and the day after bed rest to determine maximal aerobic capacity.

One week of bed rest

To simulate the impact of a standard hospitalization period, subjects underwent a 7-day period of strict bed rest. From the start of bed rest at 8:00 AM on day 1 until day 8 (test day 2), subjects were not allowed to leave the bed. During daytime, subjects were allowed to use a pillow and slight elevation of the bed-back to be able to perform their daily activities. All washing and sanitary activities were performed whilst being in the bed. Each morning subjects were woken at 7:30, and lights were switched off at 23:30. Subjects were continuously monitored by members of the research team.

Dietary intake

During the screening visit, resting energy expenditure was measured by indirect calorimetry using an open-circuit ventilated hood system (Omnical, Maastricht University, Maastricht, the Netherlands; (Schoffelen *et al.*, 1997)). During the seven days prior to bed rest, and during the bed rest period itself, dietary intake was entirely controlled. During the 7-day run-in period, subjects received all food products from the research team and prepared their meals at home. When participants reported to be hungry or overfed, energy intake was adjusted accordingly. Energy requirements were estimated based on basal metabolic rate measured during indirect calorimetry measurements, multiplied by an activity factor of 1.60 (run-in) and 1.35 (bed rest). Macronutrient composition of the diet was identical between the run-in and bed rest period. During bed rest, food administration was performed via a nasogastric tube (Flocare© PUR tube Enlock, Ch8, 110 cm, Nutricia Advanced Medical Nutrition, the Netherlands) to study the effect of dietary feeding pattern on muscle protein metabolism (Dirks *et al.*, 2019). For that

reason, subjects were fed in either a bolus pattern ($n=10$, four boluses per day, administered at 8:00 (30% of total daily food intake), 13:00 (30%), 18:00 (30%), and 23:00 (10%)) or a continuous manner ($n=10$, $\sim 100 \text{ mL}\cdot\text{h}^{-1}$, 24 h per day). Subjects were given a standard enteral food product (Nutrison Multi Fibre, Nutricia Advanced Medical Nutrition), composed of 47 en% carbohydrates, 34 en% fat, 16 en% protein, and 3 en% fibres. Both test days commenced at 8:00 AM, after an 8-hour overnight fast from 0:00 the evening before.

Insulin sensitivity

Before and immediately after one week of bed rest, after the collection of a fasted muscle biopsy sample (described below), a one-step hyperinsulinemic-euglycemic clamp was performed to assess glucose infusion rate, indicative of peripheral insulin sensitivity. We have previously reported glucose infusion rates as part of a larger clinical trial on these individuals (Dirks *et al.*, 2019). Prior to the start of the experiment, a Teflon cannula was inserted anterogradely in an antecubital vein of the forearm for the infusion of 20% glucose (Baxter B.V., Utrecht, the Netherlands) and insulin ($40 \text{ mU}\cdot\text{m}^{-2}\cdot\text{min}^{-1}$; Novorapid, Novo Nordisk Farma, Alphen aan den Rijn, the Netherlands). A second cannula was inserted in a superficial dorsal hand vein of the contralateral hand. From this cannula, arterialized venous blood was obtained by heating the hand in a hot box at 60°C . Every 5 min during the clamp, 1 mL blood was drawn for the determination of blood glucose concentration (ABL800 Flex, Radiometer Medical, Denmark). The amount of glucose infused was altered to maintain euglycemia at $5.0 \text{ mmol}\cdot\text{L}^{-1}$. Prior to and during the steady-state (last 30 min) of the clamp, fasting and insulin-stimulated energy expenditure and substrate oxidation were assessed by indirect calorimetry using an open-circuit ventilated hood system (Omnical). These data were used to calculate whole-body fat and carbohydrate oxidation rates, as well as metabolic flexibility, as described before (Most *et al.*, 2014).

Body composition

On both test days, a DXA-scan (Dual Energy X-Ray Absorptiometry; Hologic, Discovery A, QDR Series, Bedford, MA, USA) was made to assess body composition. The system's software package Apex version 4.0.2 was used to determine whole-body and regional lean mass, fat mass, and bone mineral content.

Skeletal muscle tissue collection and processing

Prior to and immediately following bed rest, a single muscle biopsy sample was collected from *m. vastus lateralis* under fasting conditions. After local anesthesia was induced, a percutaneous needle biopsy was taken approximately 15 cm above the patella using the Bergström technique (Bergstrom, 1975). The collected muscle tissue was freed from any visible blood and non-muscle tissue, and rapidly divided into two pieces. The first part was used to generate permeabilized muscle fibres, as described below, while the second part of muscle tissue was rapidly frozen in liquid nitrogen, and subsequently stored at -80°C until analyses for reduced glutathione (GSH), oxidized glutathione (GSSH), and Western blotting.

Muscle tissue analyses

Mitochondrial enzyme activity

Maximal citrate synthase (CS) activity was measured as performed previously (Wall *et al.*, 2015). In short, ~35 mg of the muscle was homogenised in 40 vol/wt of a tris buffer (250 mM sucrose, 10 mM tris, 2 mM EDTA, pH 7.4). The maximal CS activity was assayed spectrophotometrically at 37°C by measuring the rate of absorption at 412 nm in the presence of 10 mM oxaloacetate, 3 mM acetyl-CoA and 1 mM 5, 5'-dithiobis-(2-nitrobenzoate) (DTNB) (Sreere, 1969).

Mitochondrial bioenergetics

We have previously reported in detail our methodology (Holloway *et al.*, 2018). In short, a small piece of muscle tissue was immediately placed in ice-cold BIOPS (50 mM MES, 7.23 mM K₂EGTA, 2.77 mM CaK₂EGTA, 20 mM imidazole, 0.5 mM DTT, 20 mM taurine, 5.77 mM ATP, 15 mM PCr, and 6.56 mM MgCl₂·H₂O; pH 7.1), separated under a light microscope into bundles using fine-tipped forceps. The small sample chosen for one individual appeared of poor quality once under the microscope, and therefore not utilized for respiration experiments. All other fibres were treated with 30 µg·mL⁻¹ saponin for 30 min at 4°C, and washed for 15 min in buffer Z (105 mM K-Mes, 30 mM KCl, 1 mM EGTA, 10 mM K₂HPO₄, 5 mM MgCl₂·H₂O, 0.005 mM pyruvate, 0.002 mM malate with 5 mg·mL⁻¹ fatty acid-free BSA; pH 7.4). Thereafter, O₂ consumption and H₂O₂ emission were determined in buffer Z supplemented with 20 µM Amplex Red (Invitrogen, Carlsbad, CA, USA), 5 U·mL⁻¹ horseradish peroxidase, 40 U·mL⁻¹ superoxide dismutase (SOD) and 25 µM blebbistatin, using an Oxygraph-2 K with a fluorometry modular attachment (Oroboros Instruments, Innsbruck, Austria). Experiments were conducted at 37°C and commenced with mild hyper-oxygenation to prevent re-oxygenation during an experiment (250 µM O₂), in the presence of various substrates, including 10 µM cytochrome *c* to ensure outer mitochondrial membrane integrity. Importantly, in all experiments cytochrome *c* stimulated respiration < 10% (pre: +2.9±0.7%, post: 1.4±1.3%). The rate of H₂O₂ emission was calculated from a standard curve established with the same reaction conditions using the DatLab software (Oroboros Instruments) after subtracting the fibre background. All fibres were weighed in buffer Z before the start of an experiment to normalize respiration to muscle bundle weight.

Western blotting

Permeabilized muscle fibre bundles were recovered from the Oroboros system, digested, separated using SDS-PAGE, and transferred to polyvinylidene difluoride (PVDF) membranes, as described previously (Herbst *et al.*, 2014). Commercially available antibodies were used to detect Akt (Cell Signaling, Denver MA, USA; product number – cs4691, dilution 1:1000), ANT1 (Abcam, Cambridge, USA – ab110322, dilution 1:1000), ANT2 (Abcam – ab118076, dilution 1:2000), AS160 (Cell Signaling – cs2670, dilution 1:1000), GS (Cell Signaling – cs3893, dilution 1:1000), HK II (Cell Signaling – cs2867, dilution 1:1000), OXPHOS (Mitosciences, Eugene, USA – ab110413, dilution 1:500), VDAC (Abcam – ab14734, dilution 1:1000), α -tubulin (Abcam – ab7291, dilution 1:1000), and 4HNE (Alpha Diagnostics, San Antonio, TX, USA – HNE11-s, dilution 1:1000). All samples for each protein were loaded on the same membrane to limit variation, and Ponceau staining was used to verify constant loading. Importantly, Ponceau staining was not different between groups/timepoints and therefore data were not normalized. Western blots were quantified via chemiluminescence and the FluorChem HD imaging system (Alpha Innotech, Santa Clara, USA).

Glutathione redox status

Muscle GSH and GSSG concentrations were determined by high-performance liquid chromatograph (HPLC; LC-20AD; Shimadzu, Kyoto, Japan) coupled with a Microsorb 100-5 NH2 S250x4.6 mm HPLC column (Agilent Technologies, Santa Clara, CA, USA) as previously reported (Holloway *et al.*, 2018).

Statistics

All data are presented as means \pm SEM. Originally data were analysed with a 2-way ANOVA comparing feeding pattern and the effect of bed rest. Only main effects were observed, and therefore post-hoc comparisons were not appropriate. Thereafter, data were analysed for

changes over time (i.e. grouped feeding patten) using a two-sided paired-samples Student's t-test (pre vs post bed rest). Apparent Km values were determined using Michaelis-Menten kinetics, and IC₅₀ values using one-phase decay associations, in Prism 5 (GraphPad Inc., La Jolla, CA, USA). All other statistical analyses were performed using SPSS version 23.0 (IBM Corp., Armonk, NY, USA). Pearson correlation coefficient was used to test for significant correlation between the change in GIR and change in maximal mitochondrial H₂O₂ emission. Statistical significance was set at $P < 0.05$.

Results

Whole-body metabolism and insulin sensitivity

We first aimed to characterize the impact of our feeding intervention on whole-body metabolism using indirect calorimetry. Resting energy expenditure was neither altered following bed rest nor influenced by food intake pattern (**Table 1**). Neither bed rest nor feeding pattern influenced rates of carbohydrate or fat oxidation, but there was a tendency for an increase in fasting RER with bed rest ($P=0.078$), while VO_2 peak declined following bed rest in both feeding groups ($P<0.05$). We next examined the impact of our feeding patterns on whole-body insulin sensitivity via a hyperinsulinemic-euglycemic clamp. The glucose infusion rate (GIR) was dramatically reduced following bed rest (**Figure 1A**). Specifically, there was a main effect for bed rest attenuating GIR by ~42%, while the feeding pattern implemented did not modulate this decline (**Figure 1B**). Since the responses to bed rest were similar between feeding interventions, collapsing the data into a combined pre-post analysis revealed a similar statistical interpretation, namely that bed rest markedly impaired GIR (**Figure 1C**). When these data were adjusted for total body weight rather than lean body mass, a similar $43\pm3\%$ decline in glucose uptake was observed ($P<0.001$). The substantial decrease in whole-body glucose uptake was accompanied by 21-50% decreases in protein expression of Akt, AS160, HK, and GS (**Figure 1D**).

Mitochondrial respiration

We next examined various indexes of mitochondrial bioenergetics in permeabilized muscle fibres. While leak respiration (State II) in the absence of ADP was not altered, bed rest decreased ADP-stimulated respiration (State III) regardless of feeding pattern (**Supplemental Figure 1**). Since whole-body metabolism, GIR and maximal mitochondrial respiration were

not influenced by feeding pattern, we decided to collapse both groups to increase our statistical power to examine potential subtle changes in mitochondrial bioenergetics following bed rest. Collapsing the feeding groups into a single dataset did not alter the statistical interpretation of unaltered leak respiration (State II) or the apparent ~13% reduction in ADP-stimulated respiration (State III; **Figure 2A**). Moreover, OXPHOS protein content was similarly reduced after bed rest (**Figure 2B**), further suggesting a reduction in mitochondrial oxidative potential. While maximal CS activity displayed a non-significant reduction with bed rest (**Figure 2C**; $P=0.13$), when maximal respiration was normalized to CS the differences following bed rest were mitigated (**Figure 2D**; $P=0.73$), suggesting reductions in mitochondrial proteins contribute to the observed decreased oxidative capacity following 7 days of bed rest (Larsen *et al.*, 2012).

We next examined potential changes in mitochondrial bioenergetics across a range of ADP concentrations to examine the dynamic response to ADP. Similar to maximal respiration, ADP-stimulated respiration was lower following bed rest (**Figure 3A**) at virtually all ADP concentrations examined ($P<0.10$ for 175-4,000 μM ADP; $P<0.05$ for 6,000-10,000 μM ADP), including some indicative of skeletal muscle (i.e. 175 μM , (Phillips *et al.*, 1996; Howlett *et al.*, 1998)). Since maximal respiration appeared to decrease to a greater degree than submaximal respiration (especially 12.5-25 μM ADP) we interrogated possible changes in ADP sensitivity by normalizing the respiratory drive at each ADP concentration to maximal respiration. This approach revealed that following bed rest, respiration at submaximal ADP concentrations (500-4,000 μM ADP) was closer to the maximal oxidative potential, which also manifests in a decrease in the apparent ADP K_m ($P=0.106$, **Figure 3C**). In the present study the Michaelis-Menten curve fit was ~0.93 (**inset Figure 3B**), which is slightly lower than our previous reports. This may reflect the lower ADP concentrations used in the present study, as a previous

report has shown that mitochondria are uncoupled ($P/O < 1.0$) when ADP concentrations are below $15 \mu\text{M}$ (Lark *et al.*, 2016).

While bed rest decreased absolute respiration, bed rest did not alter the content of key proteins involved in mitochondrial ADP transport across the outer (VDAC) and inner (ANTs) mitochondrial membranes (**Figure 3D**). However, given the reduction in OXPHOS protein content, an increase in ADP transport proteins was observed when normalized to this marker of mitochondrial content (**Figure 3D**). Altogether, the preferential loss of electron transport chain proteins appears to largely account for the reduction in respiration, while the maintained/concentrated ADP transport proteins likely contribute to the conserved respiration in the presence of $12.5\text{-}175 \mu\text{M}$ ADP sensitivity.

Mitochondrial H_2O_2 emission and oxidative stress

Due to the setup of the experimental procedure, we were able to simultaneously monitor the ability of submaximal ADP concentrations to stimulate respiration and attenuate H_2O_2 emission rates in permeabilized muscle fibres using a methodology we have recently developed (Holloway *et al.*, 2018). One week of bed rest tended to increase maximal mitochondrial H_2O_2 emission in the presence of complex II linked substrates (succinate) alone (**Figure 4A**, $P=0.053$), or in combination with complex I linked substrates (pyruvate + malate; **Figure 4B**, $P=0.097$; +15%). Mitochondrial H_2O_2 emission normalized to CS retained this 15% increase following bed rest (**Figure 4C**) and since leak respiration was not altered (**Figure 2A**), the ratio of H_2O_2 emission to JO_2 consumption was increased following bed rest (**Figure 4D**). Combined these data strongly suggest the capacity of mitochondria to produce H_2O_2 is increased following bed rest, the change in maximal mitochondrial H_2O_2 emission in the presence of succinate was not correlated with the degree of insulin resistance (**Figure 4D**, $r = -0.157$, $P=0.522$), challenging the physiological importance this notion. Importantly, mitochondrial ROS was not

increased in the presence of submaximal ADP concentrations (**Figure 5A**), and when the data were fit with a one-phase exponential decay curve, a leftward shift of the curve was observed following bed rest (**Figure 5B**) and the apparent ADP IC_{50} to attenuate H_2O_2 tended to decrease (**Figure 5 inset**; $P=0.069$). Moreover, the ratio of H_2O_2 emission to JO_2 consumption in the presence of all ADP concentrations examined was not higher following bed rest (**Figure 6**), which altogether strongly suggests mitochondrial H_2O_2 emission is not increased following bed rest when modelled in the presence of ADP. In support of these submaximal ADP bioenergetic assessments, bed rest did not alter reduced (GSH) or oxidized (glutathione disulfide; GSSG) glutathione, the ratio between the two (GSH:GSSG), or 4HNE content (**Figure 7**; all $P>0.05$), suggesting the absence of redox stress.

Discussion

Short periods of muscle disuse lead to the rapid development of insulin resistance, and although the mechanisms remain poorly defined, we have recently shown that bed rest-induced insulin resistance is not accompanied by increases in skeletal muscle reactive lipid content (Dirks *et al.*, 2016). In the present study we demonstrate that short-term bed rest 1) induced insulin resistance and 2) impaired mitochondrial respiration across a range of submaximal ADP concentrations, in association with 3) a loss of electron transport chain, but not mitochondrial ADP transport, proteins. In addition, while bed rest 4) increased maximal mitochondrial H₂O₂ emission, the ability of ADP to attenuate mitochondrial ROS was improved with bed rest, and as a result there were no alterations in 5) H₂O₂ emission in the presence of ADP concentrations indicative of resting muscle, 6) the ratio of H₂O₂ emission to JO₂ consumption, and 7) markers of cellular redox stress following bed rest. Altogether, this work establishes that bed rest impairs mitochondrial respiratory function across a range of ADP concentrations, however bed rest-induced insulin resistance cannot be explained by an increase in mitochondrial H₂O₂ emission.

Insulin resistance and mitochondrial respiratory function

While the etiology of skeletal muscle insulin resistance remains unknown, mitochondrial dysfunction has been repeatedly implicated in this process. In the present study, bed rest decreased maximal mitochondrial respiration (**Figure 2A**), supporting previous reports of a reduction in respiratory function following 2 weeks of leg immobilization (Gram *et al.*, 2014), and our recent work highlighting leg immobilization rapidly impairs mitochondrial respiratory capacity in as little as 3 days (Miotto *et al.*, 2019). In the present study, the reduction in respiratory capacity coincided with a reduction in OXPHOS proteins. While it is tempting to speculate a causative relationship between these observations, our previous work has

highlighted that muscle disuse impairs respiration before a reduction in OXPHOS protein (Miotto et al., 2019), strongly suggesting rapid posttranslational modifications of the electron transport contribute to the observed impairment in respiratory capacity. While mitophagy likely contributes to the subsequent reduction in mitochondrial proteins (Miotto et al., 2019), the half-life of mitochondrial proteins has been estimated to be ~14 days (Menzies & Gold, 1971), suggesting subtle changes in mitochondrial content before this time may be difficult to detect. This likely accounts for muscle disuse/detraining studies yielding inconsistent findings regarding decrements in markers of mitochondrial content within 7 days (Coyle *et al.*, 1984; Wall *et al.*, 2015; Dirks *et al.*, 2016; Larsen *et al.*, 2018; Miotto *et al.*, 2019). While these data suggest temporally oxidative phosphorylation is impaired, inducing mitophagy, in contrast, a recent report has also shown that 4 days of strict bed rest reduced mitochondrial content in the absence of a reduction in respiratory capacity (Larsen *et al.*, 2018), challenging the necessity of these events. Therefore, it remains possible that posttranslational modifications of the electron transport chain transiently increase intrinsic respiratory function (Larsen *et al.*, 2018) before a subsequent decrement is observed, and the rapid reduction in mitochondrial proteins associated with muscle disuse involves a decrease in gene transcription, as opposed to mitophagy mediated clearance. Regardless of these knowledge gaps, a consistent finding is that muscle disuse decreases mitochondrial oxidative capacity (e.g. content and/or respiration). Genetic approaches that decrease mitochondrial content promote insulin resistance (Fritah *et al.*, 2012), while conversely inducing mitochondrial biogenesis attenuates the development of insulin resistance (Benton *et al.*, 2010; Wright *et al.*, 2011). Thus, the reduction in mitochondrial content and respiratory function following bed rest likely indirectly contributes to the development of insulin resistance.

While previous literature has examined the effect of bed rest on maximal ADP-stimulated respiration (>5mM ADP; (Larsen *et al.*, 2018)), biologically skeletal muscle free ADP is orders of magnitude lower, ranging between ~25-250 μ M (Phillips *et al.*, 1996; Howlett *et al.*, 1998). In the present study, in addition to a reduction in maximal ADP-stimulated respiration, we further report the novel finding that mitochondrial respiration is attenuated across a range of submaximal ADP concentrations after bed rest (**Figure 3**). It appears that the impairment in respiration was not related to a reduction in ADP transport proteins, as VDAC, ANT1 and ANT2 actually increased in relation to OXPHOS content. While the reduction in mitochondrial respiration may indirectly contribute to the induction of peripheral insulin resistance, we also demonstrate that bed rest is associated with a decrease in protein content of key proteins involved in insulin signalling (**Figure 1D**). Unfortunately, the lack of insulin-stimulated muscle biopsies precludes us from drawing conclusions on the contribution of impaired insulin signalling (or alterations in insulin-stimulated mitochondrial bioenergetics) on bed rest-induced insulin resistance (**Figure 1**). However, previous work builds on this by demonstrating that bed-rest induced insulin resistance is associated with reduced insulin-stimulated insulin signalling, which might (at least partly) explain the observed insulin resistance (Bienso *et al.*, 2012). An important limitation of all work to date is that the impact of disuse on GLUT4 translocation to the plasma membrane, which is the most adjacent physiological mechanism preceding muscle glucose uptake, is not measured. As such, the effect of impaired insulin signalling on muscle glucose uptake during disuse remains to be elucidated.

Bed rest and mitochondrial ADP sensitivity

It has been postulated that an increase in mitochondrial content could alter the sensitivity of oxidative metabolism to ADP (Holloszy & Coyle, 1984). In this manner, a reduction in mitochondrial content, as observed with bed rest, was hypothesized to attenuate mitochondrial

ADP sensitivity. However, while submaximal respiration was reduced, in contrast to our hypothesis, calculated mitochondrial ADP sensitivity was not reduced, and the relative respiration in the presence of submaximal ADP concentrations actually tended to increase (**Figure 3B, C**) in associated with reduced H₂O₂ emission (IC₅₀, **Figure 5C**). However, these values represent the concentration of ADP required to achieve half-maximal responses, and since maximal respiration was decreased (**Figure 2**) and maximal H₂O₂ emission was increased (**Figure 4**), interpreting sensitivity as 50% of the capacity of these values can be misleading when attempting to understand biology. For instance, while the sensitivity to ADP tended to be increased with bed rest, in stark contrast, absolute H₂O₂ emission rates were not altered in the presence of ADP, and respiration in the presence of non-saturating ADP was actually impaired. While the absolute responses to ADP are biologically relevant, the indices of ADP sensitivity suggest external regulation might exist that attempts to maintain metabolic homeostasis when mitochondrial content is reduced. Importantly, the opposite response occurs with exercise training, a situation associated with increased mitochondrial content and submaximal ADP-stimulated respiration rates despite a reduction in ADP sensitivity (Ludzki *et al.*, 2015). Therefore, there appears to be a complex interaction between mitochondrial content and ADP responsiveness. Although the mechanisms responsible for this remain unknown, the apparent concentration of ADP transport proteins within mitochondria may partially contribute to this response (**Figure 3D**), as exercise training appears to ‘dilute’ ANT-2 content (Ludzki *et al.*, 2015). Alternatively, ANT, which is required for the transport of ADP/ATP across the inner mitochondrial membrane, has several potential regulatory mechanisms that could influence ADP sensitivity, including acetylation of lysine 23 (Mielke *et al.*, 2014), tyrosine 194 phosphorylation (Feng *et al.*, 2008) and glutathionylation/carbonylation (Yan & Sohal, 1998; Queiroga *et al.*, 2010). While these mechanisms remain speculative, regardless, these data challenge the necessity for a reduction

in ADP sensitivity for the development of insulin resistance, and suggest the impairments in submaximal respiration are likely more biologically relevant.

Bed rest and mitochondrial H₂O₂ emission

Since submaximal respiration was impaired following bed rest, the assessment of mitochondrial H₂O₂ emission in the presence of ADP was logical. However, contrary to our hypothesis, mitochondrial ROS emission was not altered after bed rest in the presence of ADP (**Figure 5**). Moreover, the present data revealed how quickly the provision of ADP decreases mitochondrial ROS emission rates in human muscle, as the IC₅₀ value was ~15-fold lower than the apparent Km to stimulate respiration. Therefore, the increase in mitochondrial ROS emission in the absence of ADP (Gram *et al.*, 2015) represents the capacity for mitochondrial ROS production, which likely does not reflect the *in vivo* situation. This assertion is supported by the lack of correlation between the development of insulin resistance and the change in maximal mitochondrial H₂O₂ emission (**Figure 4D**). From a physiological standpoint, it is likely more appropriate to examine mitochondrial bioenergetics (respiration and ROS emission) in the presence of submaximal concentrations of ADP. Supporting this notion, while maximal ROS tended to be increased after bed rest, mitochondrial H₂O₂ emission in the presence of ADP was not altered, and neither were indexes of cellular stress (GSH:GSSG, 4HNE and protein carbonylation, **Figure 7**: present study and (Dirks *et al.*, 2016)). While we have utilized a mixture of complex I and II substrates to maximize the electron transport chain-generated H₂O₂, it is always possible that our *in vitro* protocol does not accurately reflect the *in vivo* environment. While the protocol utilized induces forward electron flow to maximize H₂O₂ production from several complexes (Holloway *et al.*, 2018), pyruvate dehydrogenase is not a producer of ROS in this protocol (Fisher-Wellman *et al.*, 2015), and we have not utilized fatty acids, and therefore our data may underestimate the production of ROS following bed

rest. However, the unaltered GSH:GSSG and 4HNE in the present study, and previous reports of unaltered markers of redox stress (Dirks *et al.*, 2016), suggest that absence of *in vivo* redox stress following bed rest, supporting our *in vitro* data. We have also previously shown bed rest does not alter the capacity of antioxidant enzymes (Dirks *et al.*, 2016), and therefore the present data strongly indicates that because of an increased and/or maintained ability of ADP to attenuate ROS production, an increase in mitochondrial ROS emission does not contribute to the induction of insulin resistance during short-term bed rest.

Dietary feeding pattern and insulin resistance

Various clinical populations suffer from a decline in metabolic health, which increases the risk for morbidity and mortality (van den Berghe *et al.*, 2001). These patients often depend on (par)enteral nutrition, administered in a continuous or intermittent manner depending on hospital protocols, although the effect of dietary feeding pattern on insulin sensitivity remains poorly defined. Here we show that the pattern of enteral food administration does not modulate the substantial decline in insulin sensitivity observed during bed rest (**Figure 1**). This is in contrast to our hypothesis, which was based on animal work showing a greater degree of insulin resistance when food was administered in a continuous manner (Stoll *et al.*, 2012). In agreement, our mitochondrial measurements demonstrated similar changes in maximal respiration and ROS production with intermittent and continuous feeding, suggesting that substrate delivery pattern does not influence the bed rest-induced metabolic decline. Although this is of great clinical relevance, it remains to be established if these findings translate to patient populations, which are further compromised by illness (van den Berghe *et al.*, 2001), medication (Dirks *et al.*, 2015), inflammation (Cree *et al.*, 2010), and/or malnutrition (Dirks *et al.*, 2015). Obviously, in the present study we also provided food based upon individual energy requirements, thereby preventing under/overfeeding and the subsequent impact on insulin

sensitivity. As such, the present data indicate that in healthy disuse situations with adequate energy and protein intake, dietary feeding pattern does not influence the observed decline in metabolic health. Similarly, the lack of a relationship between mitochondrial ROS and insulin sensitivity may be vastly different in disease/metabolically challenged individuals, and therefore the present data only suggests mitochondrial ROS does not contribute to the induction of bed rest induced insulin resistance in healthy individuals.

Conclusions

Altogether, the present data indicates that bed rest-induced insulin resistance is associated with a reduction in mitochondrial respiration across a range of ADP concentrations, however bed-rest was not accompanied by an increase in mitochondrial H₂O₂ emission or oxidative stress. While the mechanisms underlying disuse-induced insulin resistance may therefore involve reductions in the content of mitochondria (present study), insulin signalling and GLUT4 protein content (present study and (Bienso *et al.*, 2012)), our findings clearly demonstrate that alterations in mitochondrial H₂O₂ emission and nutrient delivery pattern cannot be held responsible for the rapid development of insulin resistance in humans.

Additional Information

Competing Interests: None of the authors disclose any conflicts of interest.

Author Contributions: MLD, LJCvL, and GPH designed the study. MLD, GHG, JMS, HLP, JvK, and GPH organized and performed the experiments. MLD, PMM, HLP, and GPH analysed the data. MLD, PMM, GHG, JMS, HLP, JvK, LJCvL, and GPH interpreted the data. MLD and GPH drafted and edited the manuscript, and all authors approved the final version. MLD was the guarantor of the study, had full access to all the data in the study and takes full responsibility for the integrity of the data and the accuracy of the data analysis. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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Tables

Table 1: Subjects' characteristics

	Bolus		Continuous	
	Pre	Post	Pre	Post
Basic characterization				
Age	27 ± 1		24 ± 1	
Weight	78.2 ± 5.1	77.6 ± 5.1 *	77.0 ± 5.4	76.7 ± 5.4 *
Height	1.81 ± 0.03		1.79 ± 0.03	
BMI	23.5 ± 1.3		24.0 ± 1.0	
HbA1c (%)	5.2 ± 0.1		5.2 ± 0.2	
Basal metabolism				
EE (kcal/min)	1.277 ± 0.066	1.252 ± 0.067	1.235 ± 0.047	1.244 ± 0.040
RER	0.792 ± 0.012	0.824 ± 0.018 #	0.802 ± 0.012	0.817 ± 0.017 #
CHO ox (g/min)	0.080 ± 0.014	0.115 ± 0.023	0.086 ± 0.012	0.090 ± 0.013
Fat ox (g/min)	0.080 ± 0.009	0.064 ± 0.009	0.074 ± 0.007	0.072 ± 0.007
Exercise metabolism				
Maximal oxygen uptake (mg·kg·min ⁻¹)	40.3 ± 3.0	38.9 ± 2.5 *	44.8 ± 3.1	40.7 ± 2.6 *
Power output achieved (Wmax)	225 ± 16	218 ± 15 *	253 ± 26	235 ± 4 *

BMI, body mass index; CHO, carbohydrate; EE, energy expenditure; HbA1c, glycated haemoglobin; RER, respiratory exchange ratio. * Significantly different from pre-value (time effect, $P < 0.05$). # Trend for significant time effect ($P < 0.10$).

Figure legends

Figure 1: One week of strict bed rest leads to a substantial decline in insulin sensitivity in healthy, young males ($n=20$). Blood glucose concentrations were clamped at $5.0 \text{ mmol}\cdot\text{L}^{-1}$ during a one-step hyperinsulinemic-euglycemic clamp at $40 \text{ mU}\cdot\text{m}^{-2}\cdot\text{min}^{-1}$. This resulted in a comparable decline in insulin sensitivity (i.e. glucose infusion rates (GIR)) between the bolus ($n=10$) and continuous ($n=10$) fed groups during the 2.5 h clamp procedure, performed prior to and after one week of bed rest (**A**). As there were no differences in insulin sensitivity between bolus and continuous feeding (**B**), groups were collapsed to demonstrate that insulin sensitivity had declined by $42\pm 2\%$ following one week of strict bed rest (**C**). Values represent means \pm SEM. * Significantly different from pre-bed rest value ($P<0.001$). Panel **D** represents protein expression of key proteins in the insulin signalling pathway.

Figure 2: One week of strict bed rest leads to a substantial decline in mitochondrial oxidative capacity. Mitochondrial respiratory capacity was determined in permeabilized skeletal muscle fibres taken before (pre) and after (post) one week of strict bed rest in the absence (State II) and presence (State III) of ADP (**A**). Pyruvate, malate and succinate were utilized as substrates for these measurements, while maximal respiration also included glutamate. Figure **B** displays total protein content of the oxidative phosphorylation complexes, **C** displays maximal citrate synthase (CS) enzymatic activity, while **D** normalizes maximal respiration to CS. JO_2 , mitochondrial O_2 flux; RCR, respiratory control ratio. Values represent means \pm SEM ($n=19$). * Significantly different from pre-bed rest value ($P<0.05$). # Trend for a difference when compared with pre-bed rest values ($P<0.10$).

Figure 3: One week of strict bed rest does not impair mitochondrial ADP sensitivity. ADP titrations to measure mitochondrial oxygen consumption in permeabilized muscle fibres collected before (pre) and after (post) one week of strict bed rest in the presence of pyruvate, malate and succinate. Absolute mitochondrial oxygen consumption was decreased (**A**), but relative respiration was increased (**B**), following bed rest. Relative respiration followed a typical Michaelis-Menten kinetic curve (**B inset**), and illustrated a trend for a reduction in the apparent K_m for ADP ($P=0.106$; **C**). Protein content of ADP transport proteins, voltage-dependent anion channel (VDAC) and adenine nucleotide translocase (ANT) 1 and 2, did not change with bed rest (**D**); however, its abundance relative to OXPHOS protein content did increase. Values represent means \pm SEM ($n=19$). # Trend for a significant difference when compared with pre-bed rest values ($P<0.10$). * Significantly different from pre-bed rest values ($P<0.05$).

Figure 4: Absolute maximal ROS emission in the presence of succinate (**A**) and succinate + pyruvate/malate (**B**) before (pre) and after (post) a week of strict bed rest. Maximal ROS (succinate+pyruvate/malate) normalized to CS was increased ($P<0.05$) following bed rest (**C**), as was the ratio of H_2O_2 emission to JO_2 consumption in the absence of ADP (**D**). The change in maximal mitochondrial ROS emission in the presence of succinate was not correlated with the change in glucose infusion rate (GIR, panel **E**, $r = -0.157$, $P=0.522$). Values represent means \pm SEM ($n=19$). # Trend for a significant difference when compared with pre-bed rest values ($P<0.10$). * Significantly different from pre-bed rest values ($P<0.05$).

Figure 5: Mitochondrial H_2O_2 emission decreases with increasing ADP concentrations in young participants before (pre) and after (post) one week of strict bed rest in the presence of pyruvate, malate and succinate (**A**). ADP titrations were performed to measure the attenuation

of H₂O₂ emission in permeabilized muscle fibres (**B**), and used to determine the IC₅₀ (**B inset**). Values represent means±SEM (*n*=19). # Trend for a significant difference when compared with pre-bed rest values (*P*<0.10).

Figure 6: The ratio of H₂O₂ emission to JO₂ consumption, determined by simultaneous measurement of mitochondrial respiration and H₂O₂ emission at increasing ADP concentrations, was not altered in healthy, young males (*n*=20) following one week of strict bed rest. The inset displays the ratio of H₂O₂ emission to JO₂ consumption between 0 and 250 μM ADP. * Significantly different when compared with pre-bed rest values (*P*<0.05).

Figure 7: Reduced (GSH; **A**) and oxidized (GSSG, **B**) glutathione concentrations, as well as the GSH:GSSG ratio which serves as a marker for *in vivo* redox status (GSH:GSSG, **C**), were not altered following short-term bed rest in healthy, young participants. Figure **D** depicts protein expression of 4-Hydroxynonenal (4HNE). Values represent means±SEM (*n*=19).