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Metabolic adaptations in skeletal muscle after 84 days bed rest with and without concurrent flywheel resistance exercise

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ABSTRACT

As metabolic changes in human skeletal muscle after long-term (simulated) spaceflight are not well understood, this study examined the effects of long-term microgravity, with and without concurrent resistance exercise on skeletal muscle oxidative and glycolytic capacity. Twenty-one men were subjected to 84 days head-down tilt bed rest with (BRE; n=9) or without (BR; n=12) concurrent flywheel resistance exercise. Activity and gene expression of glycogen synthase, glycogen phosphorylase (GPh), hexokinase, phosphofructokinase-1 (PFK-1) and citrate synthase (CS), as well as gene expression of succinate dehydrogenase (SDH), vascular endothelial growth factor (VEFG), peroxisome proliferator–activated receptor gamma coactivator-1 (PGC-1α) and myostatin, were analysed in samples from m. vastus lateralis collected before and after bed rest. Activity and gene expression of enzymes controlling oxidative metabolism (CS, SDH) decreased in BR, but were partially maintained in BRE. Activity of enzymes regulating anaerobic glycolysis (GPh, PFK-1) was unchanged in BR. Resistance exercise increased the activity of GPh. PGC-1α and VEGF expression decreased in both BR and BRE. Myostatin increased in BR, but decreased in BRE, after bed rest. The analyses of these unique samples indicate that long-term microgravity induces marked alterations in the oxidative, but not the glycolytic, energy system. The proposed flywheel resistance exercise was effective in counteracting some of the metabolic alterations triggered by 84-d bed rest. Given the disparity between gene expression vs. enzyme activity in several key metabolic markers, post-transcriptional mechanisms should be explored to fully evaluate metabolic adaptations to long-term microgravity with/without exercise countermeasures in human skeletal muscle.

Keywords: microgravity, glucose metabolism, eccentric-overload, spaceflight.

INTRODUCTION

The consequences of microgravity on human skeletal muscle contractile content, function and morphology are well documented (1, 19, 37, 51). In contrast, microgravity-induced alterations of skeletal muscle metabolic processes are less understood. In space-flown rats, past studies reported reduced oxidative capacity of skeletal muscle (4, 50), and investigations using animal models of unloading (i.e. hindlimb suspension) showed decreased mitochondrial enzyme activity, predominantly in fast-type muscles, together with an unchanged or even increased activity of glycolytic enzymes in skeletal muscle (46, 50). In humans, long periods of unloading seem to induce a shift towards a glycolytic muscle type (i.e. increased preponderance of type II fibers) (55). Indeed, the few studies analyzing unloading-induced metabolic changes in human skeletal muscle showed decreased citrate synthase (CS) activity, reduced capillary and mitochondrial density (5, 20), and increased phosphofructokinase (PFK) mRNA expression (12). Apart from alterations in metabolic enzymes, the expression of key molecular markers controlling mitochondrial biogenesis (peroxisome proliferator-activated receptor gamma coactivator-1; PGC-1α) and angiogenesis (vascular endothelial growth factor; VEGF) is reduced after 3-5 weeks of unloading (8, 12). This may further compromise the capacity of skeletal muscle to produce energy through oxidative metabolism. While these data support the notion of an enhanced reliance upon glycolysis at the expense of diminished oxidative potential after several weeks of microgravity exposure, the metabolic consequences of extended periods (i.e. months) of unloading in human skeletal muscle remain to be elucidated. Such information is critical in order to plan future long-term, exploratory space missions.

84 To counteract the deleterious effects of microgravity in skeletal muscle health and
85 function, different exercise interventions (resistance exercise; aerobic exercise, whole
86 body vibration) have been proposed (48, 54). According to the traditional understanding
87 of exercise adaptations, high-volume, low-intensity aerobic exercise would be efficient
88 in counteracting metabolic changes in skeletal muscle (16). However, low-volume,
89 high-intensity aerobic exercise preserved oxidative potential in astronauts on the
90 International Space Station, as indicated by maintained peak oxygen uptake, to a greater
91 extent than protocols calling for larger exercise volume (33). Even more surprising,
92 high-intensity, low-volume flywheel RE, originally designed to combat compromised
93 muscle force and size during microgravity (3, 53), was sufficient to rescue the decreased
94 expression of PGC-1α and VEGF after 5 week unilateral lower limb suspension (12).
95 These results infer augmented muscle adaptability in response to unloading, regardless
96 of the stimulus imposed. While intriguing, such hypothesis needs to be tested during
97 systemic, long-term unloading.
98
99 In an attempt to reveal the long-term microgravity-induced skeletal muscle metabolic
100 alterations in humans, we examined muscle samples from subjects who had undertaken
101 84 days of bed rest, with or without concurrent flywheel RE. Previously, we and others
102 have reported changes in muscle volume and function (3, 44, 45), and single muscle
103 fiber properties (15, 55) in this population. The specific aim of the current study was to
104 investigate skeletal muscle oxidative and glycolytic capacity in response to 84 days
105 head-down tilt bed rest with or without concurrent flywheel RE. Given our previous
106 results employing flywheel RE in unloaded muscle (12), we hypothesized that i) long-
107 term bed rest would reduce enzyme activity and molecular factors regulating oxidative
108 energy processes, with a concomitant increase in the activity of glycolytic enzymes; and

ii) flywheel RE performed during bed rest would partly counteract such alterations. In addition, both gene expression and activity of oxidative and glycolytic enzymes were analyzed, allowing for an examination of the mechanisms controlling long-term, unloading-induced metabolic alterations in human skeletal muscle.

MATERIALS AND METHODS

General design

Twenty one men (26-41 yr) were assigned to either bed rest with (BRE; n=9) or without (BR; n=12) concurrent iso-inertial RE (4 sets of 7 maximal concentric-eccentric repetitions every third day) for the quadriceps muscle employing flywheel technology. Muscle biopsies from m. vastus lateralis were obtained from all subjects before bed rest and prior to reambulation. Activity and mRNA content of enzymes controlling aerobic and anaerobic glucose metabolism were assessed; i.e. CS, PFK-1, glycogen synthase (GS), glycogen phosphorylase (GPh), and hexokinase (HK). In addition, gene expression of succinate dehydrogenase (SDH) subunits (A, B, C, D), PGC-1α, VEGF and myostatin was also evaluated.

Subjects

Potential candidates were interviewed by trained personnel from the Institute for Space Medicine and Physiology (MEDES) clinic, where the study was carried out. After a general physical examination, twenty one healthy men were recruited and assigned to either 90 days of head-down tilt bed rest with (BRE; n=9, 33±5 yr, 176±5 cm, and 71±6 kg) or without (BR; n=12, 32±4 yr, 173±3 cm, and 72±5 kg) concurrent flywheel RE. Subjects were informed of the purposes, risks and premises associated with the study before written consent for participation was obtained. The study was conducted in accordance with the declaration of Helsinki, and protocols were approved by the local Ethical Committee in Toulouse (*le Comité Consultative de Protection des Personnes dans la Recherché Biomédicale de Toulouse I*).

Head-down tilt bed rest

Subjects were subjected to 6º head-down tilt position at all times (i.e. rest, shower, transportation, exercise training, toilet procedures). Yet, participants were allowed to perform movements in the horizontal plane and to rest on their elbows during meals. Actions to ensure compliance included video surveillance and pressure-sensitive mattresses. Massages and ankle circumduction movements were performed on a daily basis by physiotherapists.

Flywheel resistance exercise

The BRE group performed flywheel RE employing the supine (6º head-down tilt) squat exercise mode every third day (2-3 days per week), beginning on day 5 of bed rest. Each exercise session consisted of 4 sets of 7 maximal concentric-eccentric repetitions, with a recovery time of 2 min between sets. Joint angular velocity, joint angles, force, work and power were measured for each repetition (3).

Skeletal muscle biopsies

Muscle biopsies were obtained from the mid portion of the m. vastus lateralis of the dominant leg in all subjects before bed rest (PRE), and prior to reambulation at day 84 of bed rest (POST). Although the bed rest period lasted 90 days, POST biopsy samples were collected at day 84 to avoid any potential interference of multiple testing procedures during the last 5 days of the bed rest intervention. Under local anesthesia, 5-mm Bergström-needles (6) were used to obtain muscle tissue samples that were immediately cleansed of excess blood, fat, and connective tissue before being frozen in liquid nitrogen at -80°C.

164 **Enzymatic activity**

165 About 10 mg of muscle tissue per sample was homogenized in 30 volumes of ice-
166 cooled extraction medium (50 mM HCl-Tris, 4 mM EDTA, 50 mM KF, 30 mM β-
167 mercatoethanol, pH 7) and centrifuged at 15,000 g at 4ºC for 15 min. Then, enzymatic
168 activities were measured immediately in the supernatant. GS and GPh were measured
169 by radioactive methods (22). HK (total), PFK-1 and CS were determined by
170 spectrophotometry as previously described (36). Total protein concentration was
171 measured using Bradford's technique (7) to express the enzymatic activity as mU/mg of
172 protein.

173

174 **RNA isolation, reverse transcription, and real-time PCR**

175 Total RNA from muscle tissue samples (~20 mg wet weight) was extracted using
176 TRIZOL® (Invitrogen Life Technologies, Carlsbad, CA). Reverse transcription into
177 cDNA was performed on 1 μg of total RNA from each sample following the
178 instructions of a commercial kit (High Capacity Reverse Transcription Kit, Applied
179 Biosystems, Foster City, CA). Real-time polymerase chain reaction (PCR) was used to
180 measure specific mRNAs (ABI-PRISMA 7700 Sequence Detector, Perkin-Elmer
181 Applied Biosystems). TaqMan® primers and probes for myostatin (Hs00193363_m1),
182 PGC-1α (Hs01016724_m1), VEGF (Hs99999070_m1) and PFK (Hs00175997_m1)
183 were derived from the TaqMan® Gene Expression Assays (Applied Biosystems). Roche
184 Universal probe library guidelines were followed to design the amplicons for SDH
185 subunits A, B, C, and D genes, using short hydrolysis probes: HK-2 (HK-2; UPL#69.
186 CATTTTGCCAAGCGTCTACA, CTTTGCCACTGCCATCCT); CS (CS; UPL#66,
187 TCCGACCCTTACCTGTCCTT, ACTTCCTGATTTGCCAGTCC); GS (GYS1;
188 UPL#2 2, TATGAGCCTTGGGGCTACAC, GGTCTGCGATGTGTTCCTC); GPh

(PYGM; UPL#87, CTCGTGTCCTGTACCCCAAT, TTGAAGCGACGGATGATGT); SDH A (SDHA UPL#5. ATTTGGTGGACAGAGCCTCA, CTGGTATCATATCGCAGAGACCT); SDH B (SDHB; UPL#5, GGTCGCCCTCTCCTTGAG, GATGGCAAATTTCTTGATACGG); SDH C (SDHC; UPL#57, TGGAACTTGTGAAGTCCCTGT, TTTTCCTAGGTCCCACATCAA); SDH D (SDHD; UPL#57, CAGCCCTCACTCTTCATGGT, AGCTTTCTGCAAGGCATCC). Amplification mixes (10 µl) contained the diluted (1:100) cDNA sample (4.5 µl), 2x TaqMan® Fast Universal PCR Master Mix (5.0 µl) and specific primers (0.5 µl). Reactions performed with Roche Universal probe library consisted of 10µl 2X Lightcycler 480 probes mastermix (Roche #4707494001), 0.2µl corresponding probe 100X, 1µl of each primer 10µM, 5 µl of the diluted cDNA sample and PCR-grade water to 20µl. Thermal cycling protocol employed consisted of 2 min at 50°C and 10 min at 90°C followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Gene expression levels were determined using the 2-ΔΔCT method (26, 39). Any potential variation in RNA loading and quantification was corrected by employing 18S rRNA (UPL#66, ATCCATTGGAGGGCAAGTC, GCTCCCAAGATCCAACTACG; TaqMan®, Hs01375212_g1) as an endogenous control. Glyceraldehyde phosphate dehydrogenase (GAPDH, Roche GAPD gene assay #05190541001; TaqMan®, Hs99999905_m1) was analyzed as a secondary endogenous control gene. Results were almost identical for 18S and GAPDH such that the GAPDH/18S ratio did not vary.

Data Analysis

A one way ANOVA was applied to each of the four groups of samples: PRE BR vs. PRE BRE; PRE BR vs. POST BR; PRE BRE vs. POST BRE; POST BR vs. POST BRE. To directly compare BR and BRE sets of samples, the increment non-dimensional

ratio INC = ([post_value]-[pre_value])/[pre_value] was calculated for each variable. One way ANOVA was applied to INC BR vs. INC BRE. In all ANOVA analyses, Levene's Test for Equality of Variances was performed. Welch and Brown and Forsythe tests were used when the assumption of homogeneity of variances was not met. Discriminant analysis by Wilks stepping method was used to complement mean comparisons by studying effectiveness of the 29 variables in distinguishing INC BR(13) from INC BRE(10) and observing variability. Wilks Lambda measures the proportion of the total variance not yet explained by a set of variables. It decreases from 1 at each step when a new variable is selected until no further improvement is produced. Wilks Lambda would be close to 0 at this moment. Pearson's correlation was calculated to measure the degree of linear dependence among all INC variables in BR and BRE. The significance level was set at 0.05. All statistical analyses were performed using SPSS version 18 (SPSS Inc., Chicago, IL).

Abbreviations: PGC-1α, peroxisome proliferator-activated receptor gamma coactivator-1; VEFG, vascular endothelial growth factor; GS, glycogen synthase; GPh, glycogen phosphorylase; HK-2, hexokinase-2; PFK-1, phosphofructokinase-1; CS, citrate synthase; SDH A, succinate dehydrogenase a subunit; SDH B, succinate dehydrogenase b subunit; SDH C, succinate dehydrogenase c subunit; SDH D, succinate dehydrogenase d subunit; Micro-RNA, miRNA; BR, bed rest group; BRE, bed-rest and flywheel resistance exercise group; RE, resistance exercise.

RESULTS

Briefly and as reported elsewhere in greater detailed (3), muscle volume of m. quadriceps decreased by 18% in BR after the bed rest period, while no changes were reported for BRE. Likewise, force and power decrements were much greater for BR than BRE (3). In addition, single fiber analysis revealed reduced diameter, peak force and specific force in myosin heavy chain (MHC) I fibers, and decreased peak force in MHC IIa fibers in BR, but not BRE (55).

No significant differences between BR and BRE were found at PRE in activities or gene expression levels, except for HK activity ($P = 0.03$). Given that characteristics of BR vs. BRE did not differ in terms of sex, height and body mass, the difference found in HK at PRE may be explained by a chance/random effect.

Bed rest triggered a general reduction in the activity in most of the enzymes analyzed with a statistically significant decrease in the activity of HK ($P = 0.03$) and CS ($P = 0.001$) (Table 1). Although the exercise training performed by BRE induced an increment in the activity of the majority of the enzymes evaluated (except CS activity), statistically significant changes were only obtained for GPh ($P = 0.05$) (Table 1). Analysis of pre-to-post change between BR vs. BRE (INC), showed activity of HK ($P = 0.05$) and GPh ($P = 0.006$) was significantly different across groups, indicating increased activity in BRE (Table 1 and Fig.1A).

After the 84 d of bed rest, subjects from BR showed reduced gene expression of most of the enzymes analyzed (HK-2; $P = 0.0001$, CS; $P < 0.0001$, SDHB, $P = 0.0001$, SDHC, $P = 0.036$) (Table 2). However, mRNA levels of PFK increased after 84 d bed rest ($P =$

0.05). The flywheel RE training performed by BRE preserved gene expression of CS, SDHB and SDHC, and attenuated the reduction in HK-2 mRNA levels. In addition, no significant increase in PFK expression was found in BRE (Table 2). INC analysis of PRE-to-POST change between BR vs. BRE, showed mRNA expression was significantly different for HK-2 ($P = 0.045$), CS ($P = 0.02$) and SDHB ($P = 0.008$), indicating smaller changes in BRE than BR after 84 d bed rest (Table 2 and Fig.1B).

A reduced gene expression of PGC-1α and VEGF from PRE to POST was found in BR ($P = 0.031$ and $P = 0.009$, respectively) and BRE ($P = 0.014$ and $P < 0.0001$, respectively) (Table 2). Myostatin, a negative regulator of muscle mass, increased after the 84 d of bed rest in BR ($P < 0.0001$), but decreased in BRE ($P = 0.011$). Analysis of PRE-to-POST change between BR vs. BRE (INC), showed mRNA expression of VEGF ($P = 0.04$) and myostatin ($P < 0.0001$) was different across groups, indicating a greater reduction of both VEGF and myostatin in BRE (Table 2 and Fig. 1C).

Significant correlations among the activity of all the enzymes measured in BR were identified (except PFK-1 vs. GPh, and PFK-1 vs. GS) (Table 3). Thus, Pearson's correlation coefficient was in the range of 0.57-0.82 for all pairs analyzed. Likewise, significant correlations were found between activity of all enzymes measured in BRE (r values between 0.93-0.99), except in those correlations involving GPh activity.

The discriminant analysis by the Wilks stepwise method was used to further investigate the relationships among outcomes measured. Myostatin, GPh (activity), GPh (mRNA), SDHB (mRNA), SDHD (mRNA) and HK-2 (mRNA) were the variables explaining to a greater extend the changes in BR vs. BRE. After each of the six steps, the Wilks

Lambdas were 1.00, 0.47, 0.35, 0.23, 0.18, 0.12 respectively, indicating a good classifying result (Table 4). The inclusion of more steps and variables did not improve the results.

290

291 DISCUSSION

292 This study examined the consequences of 84 d simulated microgravity on skeletal
293 muscle oxidative and glycolytic capacity, and the efficacy of flywheel RE to counteract
294 microgravity-induced metabolic alterations. Long-term head-down tilt bed rest induced
295 a decreased in the activity and/or gene expression of enzymes involved in oxidative
296 energy production (CS and SDH), along with a reduction in the gene expression of well-
297 known markers of aerobic capacity in humans (VEGF and PGC-1α). In contrast,
298 glycolytic metabolism seemed to be maintained (GPh and PFK-1 activities) or slightly
299 increased (PFK-1 mRNA). Importantly, high-intensity, low-volume flywheel RE was
300 effective in offsetting some of the microgravity-induced metabolic alterations by
301 reducing the magnitude of oxidative deconditioning. The current experiments, assessing
302 both enzyme activity and gene expression, revealed that although mRNA changes partly
303 explain the unloading-induced alterations, enzymatic activity should be measured to
304 evaluate the real impact of microgravity on skeletal muscle metabolism. It follows that
305 different post-transcriptional regulatory mechanisms should be considered and studied
306 in future investigations.

307

308 HK is a key enzyme controlling the entry and phosphorylation of glucose in skeletal
309 muscle. Although muscle HK-1 is a constitutive enzyme, the vast majority of HK
310 activity in skeletal muscle is delivered by the HK-2 isoform, which is also the isoform
311 subjected to potential changes (41). Long-term bed rest induced a decrease in HK
312 activity, together with a reduction in HK-2 gene expression levels, likely reducing the
313 potential of the entire glucose energy system. These results contrast with previous
314 reports in rats showing microgravity-induced increments of HK activity (10, 30), which

highlights the difficulty of extrapolating conclusions form studies using animal models to humans. Flywheel RE was effective in offsetting microgravity-induced HK activity alterations, as well as reducing the impact of unloading in HK-2 gene expression.

CS and SDH, with two hydrophilic (A and B) and two hydrophobic (C and D) subunits, are two of the most critical enzymes controlling oxidative energy production. The results from the current study, showing decreased mRNA levels of SDHB and SDHC, together with a reduction in CS activity and gene expression, extend previous findings of decreased skeletal muscle oxidative potential after simulated microgravity (5, 14, 23, 43), and add novel information regarding oxidative metabolism alterations after long-term microgravity exposure. In addition, 84 d bed rest triggered a reduction in the gene expression of PGC-1α and VEGF, key markers controlling mitochondrial biogenesis (59) and angiogenesis (18), respectively. The reduced PGC-1α and VEGF mRNA levels are an additional indication of compromised aerobic capacity in skeletal muscle after long-term bed rest. Our data contrast previous findings after short-term microgravity (2, 43), yet confirm results from more extended unloading studies (8,12). Flywheel RE could not compensate the unloading-induced downregulation of PGC-1α and VEGF in the current investigation, contrasting previous reports of 5 wk unilateral lower limb suspension (12). However, the current results do not rule out that expression of PGC-1α and/or VEGF could have been increased immediately after each RE bout (13, 27), yet showing an overall decrease in the POST measurements. Indeed, this notion would help explaining the preserved CS activity, and mRNA levels of CS, SDHB and SDHC in BRE subjects. In addition, the apparent disparity in exercise-induced adaptations of oxidative/aerobic molecular markers after bed rest may be explained by a slower or less sensitive response of growth/transcriptional coactivators to exercise when compared

with oxidative enzymes, as suggested elsewhere (43). In any case, the results of the current investigation seem to support the idea of an augmented muscle's responsiveness to adapt during unloading (12), given that the RE program employed could maintained the activity and expression of key oxidative enzymes even though it was not originally designed to affect muscle oxidative potential.

In an attempt to compensate for the reduced oxidative metabolic capacity, the skeletal muscle usually shows increased reliance upon glycolysis when subjected to 0-g (4, 12, 50). The current study expands this notion to long-term microgravity exposure, as indicated by the increased PFK gene expression after 84 d bed rest. Of note however, PFK activity, as well as GPh, which catalyze the rate-limiting step in the glycogenolysis, remained unchanged after the bed rest period.

The current study, assessing both activity and gene expression levels on enzymes controlling oxidative and glycolytic metabolism, allows for preliminary observations regarding the mechanisms governing the metabolic changes induced by microgravity with or without RE countermeasures. Thus, 84 d bed rest induced changes in mRNA levels that were followed by the consequent, same-direction alteration in enzyme activity (GS, HK and CS). Yet, PFK gene expression increased significantly, while their activity tended to decreased after bed rest. The relationships between activity vs. gene expression seems to be altered by the RE protocol, where a mismatch in these outcome measurements was found for GS and HK. A potential explanation for these divergences is a different time course of gene expression vs. enzyme activity. The results suggest that activities of both GS and HK may be specifically degraded during immobilization or that mRNAs are less stable than proteins (57). Indeed, such effect has been described

for HK, where increased mRNA preceded any change on HK activity before returning to basal levels (22, 35). In addition, changes in enzyme activity upon microgravity and/or exercise may be dependent on post-transcriptional regulatory mechanisms (i.e. microRNAs). microRNAs are single-stranded and short RNA molecules that bind to their specific mRNA target repressing its corresponding protein expression, with a significant role in exercise-induced muscle adaptations (47). Clearly, there is a need for studies investigating the potential role of microRNAs in metabolic alterations under 0-g conditions. In any case, it appears that to evaluate metabolic alterations to microgravity with and without concurrent exercise protocols, gene expression analysis may not be accurate enough, making the assessment of end-point enzyme activity adaptations, necessary.

The correlation analysis performed in the current study revealed that microgravity-induced alterations in the metabolic systems of skeletal muscle are highly coordinated. Likewise, the impact of flywheel RE on oxidative and glycolytic capacity seems to be tightly harmonized, with the exception of GPh activity. Indeed, it appears that, for unknown reasons, GPh over-responds to flywheel RE when compared with the activity of other enzymes analyzed. In addition, our statistical approach indicated that changes in myostatin were the most powerful variable differentiating BR from BRE subjects, i.e. effect of the exercise countermeasure. This is not surprising, given that myostatin is a negative master regulator of muscle mass, and the differences in muscle size adaptations were very significant across groups in the present sample due to the exercise paradigm employed (3). Yet, several metabolic markers (GPh activity and gene expression, and mRNA levels of SDHB, SDHD and HK-2) also help to identify the effect of the exercise countermeasure proposed.

390 Furthermore from the prominent role of myostatin in muscle mass regulation, there is an
391 extensive amount of evidence relating myostatin and muscle energetic metabolism.
392 Exposure of muscle to β2-adrenergic stimulation, a hypertrophic stimulus, modifies
393 expression levels of several genes associated with myostatin signaling, attenuating its
394 effects, while enhancing HK-2 expression in muscle (38). Furthermore, *in-silico*
395 analysis of the promotor regions of HK-2, SDH B and myostatin genes using the
396 PROMO algorithm (31) revealed several common putative transcription factors binding
397 sites, including glucocorticoid receptor motive. Interestingly, glucocorticoids in skeletal
398 muscle induce protein degradation, likely involving myostatin, while attenuating insulin
399 signaling (24), which regulates HK-2 expression (58). Moreover, myostatin enhances
400 glycolysis via AMPK activation (9). Likewise, near total ablation of myostatin signaling
401 caused by genetic depletion or specific signaling blockade (49, 40, 32, 42) elicits a
402 decrease in oxidative metabolism. In contrast, a modest decrease of myostatin signaling
403 enhances oxidative metabolism (34), as was also observed in response to aerobic
404 exercise (21). Although the mechanism(s) relating myostatin and metabolic enzymes
405 expression is not well understood, the evidence supports a role of myostatin in the
406 regulation of energy metabolism, either directly or indirectly. Therefore, future studies
407 should insure the potential role of myostatin as a valuable biomarker to evaluate the
408 effect of a particular exercise paradigm or disuse condition on skeletal muscle,
409 including both muscle mass and the oxidative capacity in the muscle.

410 More systematic research is needed to elucidate whether flywheel RE could be a
411 realistic model to prevent skeletal muscle metabolic alterations in a 0-g environment.
412 While results from the current investigation indicate this may be partly true, it is
413 unlikely this RE model would succeed in counteracting spaceflight cardiovascular
414 deconditioning (25, 56). To this end, our group has developed and validated both

exercise tools and protocols using iso-inertial flywheel technology (11, 13, 28, 29, 52), which allow for both resistance and cardiovascular exercise, to meet operational aspects of serving future long-term, interplanetary space missions.

In conclusion, this study demonstrated that 84 d microgravity exposure compromise skeletal muscle oxidative potential by reducing the activity and/or gene expression of master regulators of aerobic metabolism, i.e. CS, SDH, and PGC-1α. In contrast, glycolytic capacity was essentially unaltered (GPh, PFK). As we hypothesized, high-intensity, low-volume flywheel RE was effective in counteracting some, but not all, unloading-induced metabolic alterations. Thus, it appears other exercise paradigms, most likely combining aerobic and resistance exercise, should be used for complete protection against microgravity-induced skeletal muscle alterations. Additionally, our experiments indicate that the regulation of muscle metabolic function is rather complex, with several potential post-transcriptional mechanisms involved in the final end-point adaptations (i.e. enzymatic activity). This novel information advances our understanding to enhance in-flight exercise hardware and protocols for future long-haul space missions.

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640 **Figure Captions**

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642 **Figure 1.** Non-dimensional ratio (INC = ([post_value] - [pre_value]) / [pre_value]) of
643 A, enzyme activity; B, gene expression (mRNA) of enzymes; and C, gene expression of
644 growth factors and transcriptional coactivators, for BR (84 d bed rest, grey bars, □)
645 and BRE (84 d bed rest with flywheel resistance exercise, black bars, ■). CS; citrate
646 synthase, PFK; phosphofructokinase, HK; hexokinase, GPh; glycogen phosphorylase,
647 GS; glycogen synthase, SDH A; succinate dehydrogenase a subunit, SDH B; succinate
648 dehydrogenase b subunit, SDH C; succinate dehydrogenase c subunit, SDH D;
649 succinate dehydrogenase d subunit, VEGF; vascular endothelial growth factor, PGC-1α;
650 peroxisome proliferator–activated receptor gamma coactivator-1. *: denotes BR vs BRE
651 difference at $P < 0.05$ level; ***: denotes BR vs. BRE difference at $P < 0.001$ level.

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667 **TABLES**

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Table 1. Enzymatic activities of subjects undergoing 84 d bed rest with (BRE; n = 9) or without (BR; n = 12) flywheel resistance exercise.

	PRE	POST	P (PRE-POST)	INC	P (INC_BR vs INC_BRE)
Glycogen synthase					
BR	23.6 ± 12.2	17.7 ± 9.6	0.20	-0.14	0.14
BRE	18.8 ± 10.0	23.4 ± 9.5	0.33	0.60	
Glycogen phosphorylase					
BR	265.4 ± 173.2	254.1 ± 164.7	0.87	-0.02	0.006***
BRE	205.1 ± 91.7	301.7 ± 121.3	0.05*	0.54	
Hexokinase					
BR	13.3 ± 3.3	10.6 ± 2.0	0.027*	-0.17	0.05*
BRE	8.6 ± 3.9	10.6 ± 4.4	0.33	0.43	
Phosphofructokinase					
BR	638.4 ± 209.2	598.2 ± 163.6	0.60	0.01	0.17
BRE	449.5 ± 245.4	555.7 ± 230.4	0.35	0.66	
Citrate synthase					
BR	84.8 ± 17.1	60.34 ± 15.1	0.0012***	-0.27	0.24
BRE	69.8 ± 41.4	60.07 ± 26.6	0.56	0.21	

Activities are expressed as mU/mg of protein. Values are means ± SD. *; P < 0.05, **; P < 0.01, ***; P < 0.001.

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Table 2. Gene expression (mRNA) of selected enzymes and growth/transcriptional factors of subjects undergoing 84 d bed rest with (BRE; n = 9) or without (BR; n = 12) flywheel resistance exercise.

	PRE	POST	P (PRE-POST)	INC	P (INC_BR vs INC_BRE)
Glycogen synthase					
BR	20.04 ± 5.7	17.28 ± 4.5	0.20	-0.09	0.46
BRE	18.24 ± 4.0	17.35 ± 4.0	0.64	0.01	
Glycogen phosphorylase					
BR	52.82 ± 11.4	53.8 ± 22.7	0.89	0.06	0.32
BRE	50.52 ± 16.8	59.87 ± 18.2	0.27	0.24	
Hexokinase 2					
BR	8.99 ± 3.7	3.37 ± 2.0	0.0001***	-0.60	0.045*
BRE	9.02 ± 2.6	5.10 ± 1.3	0.001***	-0.41	
Phosphofructokinase					
BR	47.47 ± 10.	59.98 ± 18.5	0.050*	0.32	0.94
BRE	43.48 ± 11.7	55.28 ± 15.2	0.08	0.34	
Citrate synthase					
BR	69.01 ± 15.7	43.33 ± 8.5	< 0.00001***	-0.33	0.02*
BRE	63.17 ± 12.9	61.47 ± 13.6	0.078	0.005	
SDH A					
BR	24.21 ± 5.9	20.13 ± 5.3	0.09	-0.14	0.61
BRE	21.89 ± 5.3	19.82 ± 6.2	0.5	-0.09	
SDH B					
BR	12.21 ± 2.3	7.63 ± 2.5	0.0001***	-0.35	0.008***
BRE	11.22 ± 2.1	10.24 ± 1.5	0.28	-0.06	
SDH C					
BR	37.03 ± 8.6	28.52 ± 9.9	0.036*	-0.19	0.20
BRE	33.10 ± 6.9	31.50 ± 6.7	0.62	-0.02	
SDH D					
BR	22.08 ± 4.0	18.24 ± 5.6	0.069	-0.15	0.21
BRE	19.48 ± 3.5	18.97 ± 3.6	0.76	0.00	

PGC-1α					
BR	0.82 ± 0.23	0.62 ± 0.25	0.031*	-0,18	0.14
BRE	0.78 ± 0.19	0.51 ± 0.10	0.014*	-0,32	
VEGF					
BR	2.90 ± 0.61	2.15 ± 1.13	0.009**	-0.26	0.04*
BRE	3.13 ± 1.08	1.66 ± 0.53	< 0.0001***	-0.45	
Myostatin					
BR	0.22 ± 0.09	0.52 ± 0.18	< 0.0001***	1.66	0.000***
BRE	0.22 ± 0.07	0.14 ± 0.04	0.011**	-0.32	

Gene expression levels are expressed as arbitrary units. Values are means ± SD. SDH A; succinate dehydrogenase a subunit, SDH B; succinate dehydrogenase b subunit, SDH C; succinate dehydrogenase c subunit, SDH D; succinate dehydrogenase d subunit, PGC-1α; peroxisome proliferator-activated receptor gamma coactivator-1, VEGF; vascular endothelial growth factor. *; $P < 0.05$, **; $P < 0.01$, ***; $P < 0.001$.

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Table 3. Pearson's correlation coefficient (r) of relative changes from PRE to POST in enzyme activity of subjects undergoing 84 d bed rest with (BRE; n = 9) or without (BR; n = 12) flywheel resistance exercise.

	BR	**BRE**
GS vs. GPh	0.68*	0.55
GS vs. HK	0.77**	0.95**
GS vs. PFK-1	0.56	0.93**
GS vs. CS	0.57*	0.94**
GPh vs. HK	0.65*	0.59
GPh vs. PFK-1	0.54	0.67
GPh vs. CS	0.79**	0.62
HK vs. PFK-1	0.75*	0.99**
HK vs. CS	0.73**	0.99**
PFK-1 vs. CS	0.82**	0.99**

GS; glycogen synthase, GPh; glycogen phosphorylase, HK; hexokinase, PFK-1; phosphofructokinase, CS; citrate synthase. *; $P < 0.05$, **; $P < 0.01$.

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Table 4. Discriminant analysis by Wilks' Lambda method applied to the one-dimensional ratio (INC = ([post_value] - [pre_value]) / [pre_value]). The right column shows the P value for the difference in INC across groups (i.e. 84 d bed rest vs. 84 d bed rest with resistance exercise).

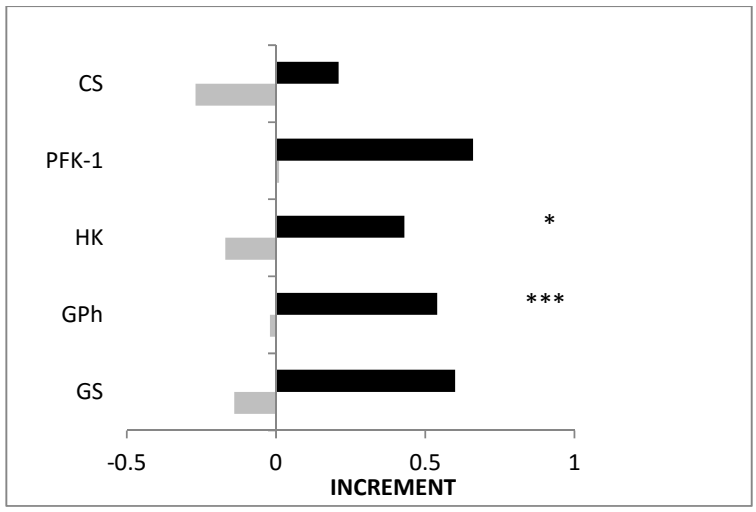
Step	1	2	3	4	5	6	P
Myostatin	1.00	0.68	0.67	0.51	0.31	0.22	< 0.0001***
GPh (activity)		0.47	0.46	0.34	0.23	0.22	0.006**
GPh (mRNA)			0.35	0.33	0.20	0.16	0.326
SDH B (mRNA)				0.23	0.22	0.18	0.008**
SDH D (mRNA)					0.18	0.15	0.854
HK-2 (mRNA)						0.12	0.046*

GPh; glycogen phosphorylase, SDH B; succinate dehydrogenase b subunit, SDH D; succinate dehydrogenase d subunit, HK-2; hexokinase 2. *; $P < 0.05$, **; $P < 0.01$, ***; $P < 0.001$.

FIGURES

Figure 1.

A.



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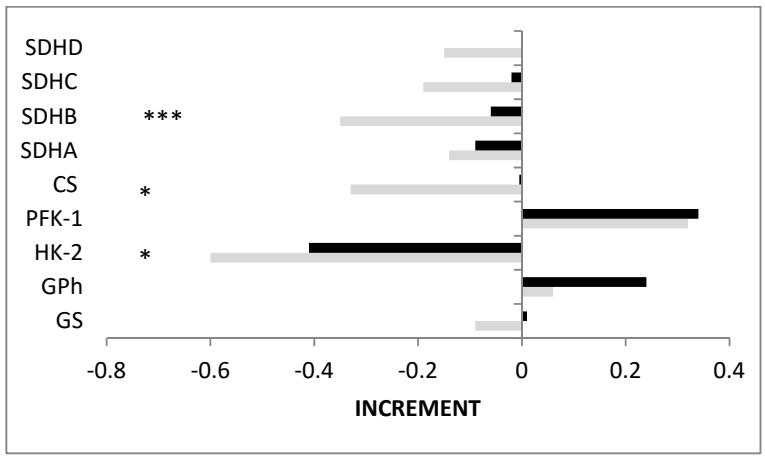
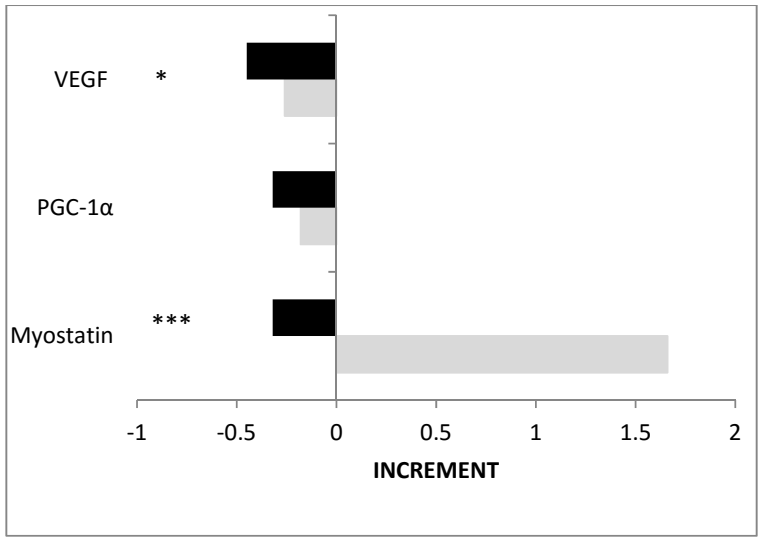


726 **C.**



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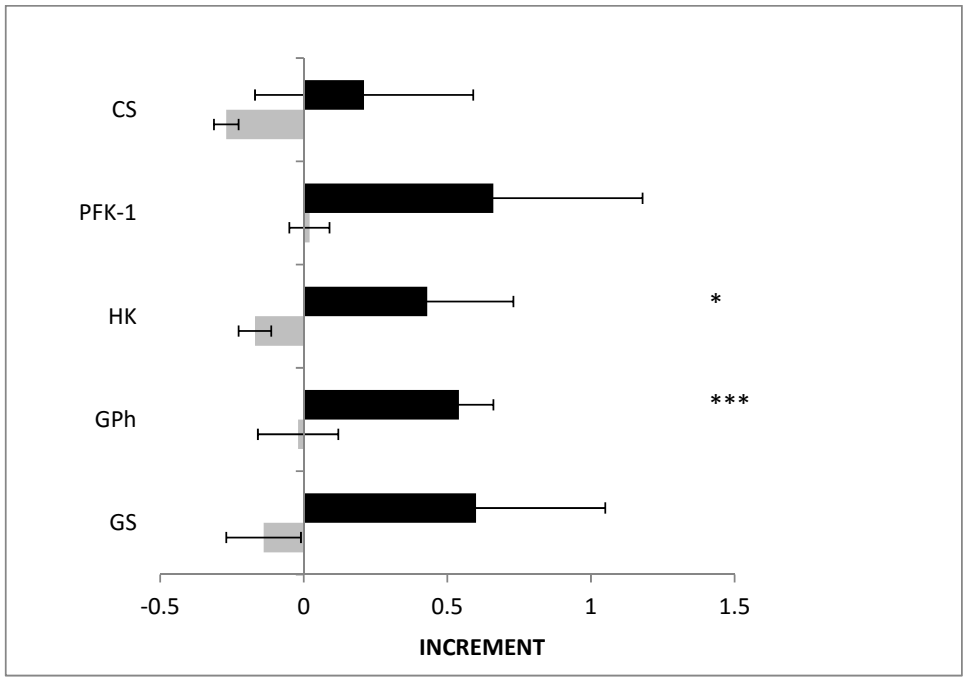
FIGURES

Figure 1.

A.



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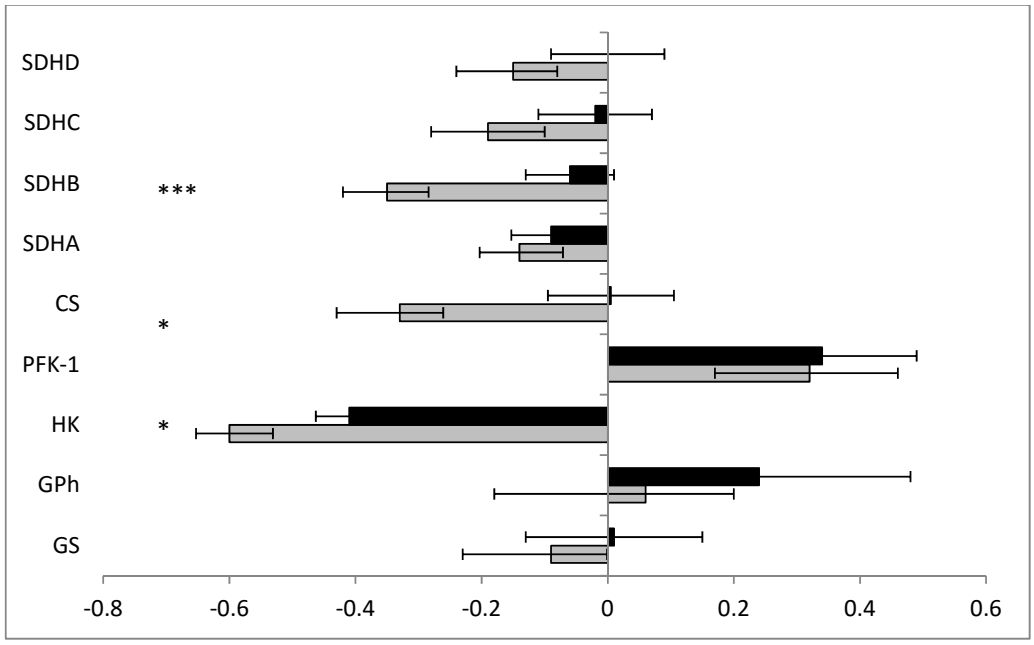
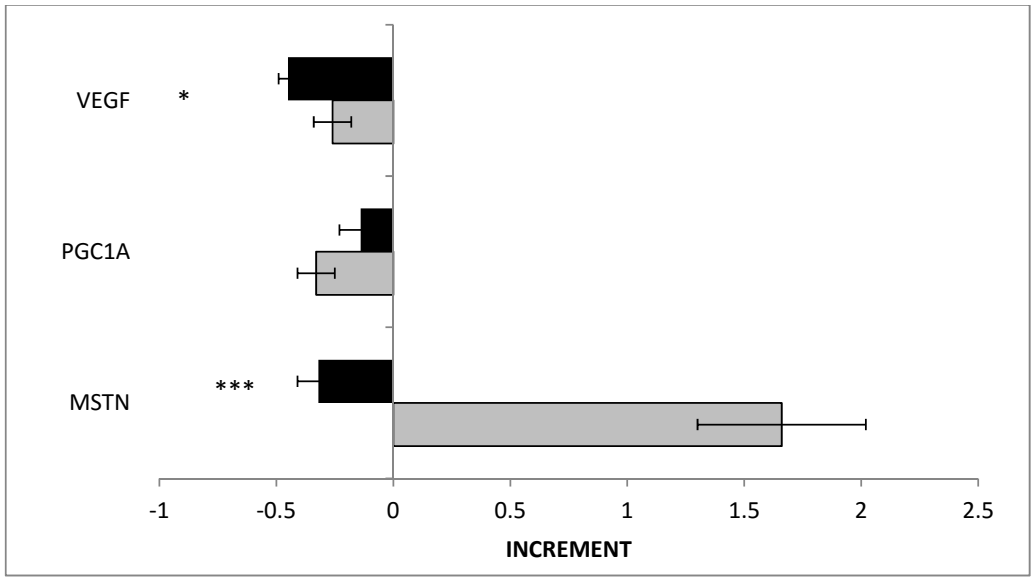


C.



TABLES

Table 1. Enzymatic activities of subjects undergoing 84 d bed rest with (BRE; n = 9) or without (BR; n = 12) flywheel resistance exercise.

	PRE	POST	P (PRE-POST)
Glycogen synthase			
BR	23.6 ± 12.2	17.7 ± 9.6	0.20
BRE	18.8 ± 10.0	23.4 ± 9.5	0.33
Glycogen Phosphorylase			
BR	265.4 ± 173.2	254.1 ± 164.7	0.87
BRE	205.1 ± 91.7	301.7 ± 121.3	0.05*
Hexokinase			
BR	13.3 ± 3.3	10.6 ± 2.0	0.027*
BRE	8.6 ± 3.9	10.6 ± 4.4	0.33
Phosphofructokinase-1			
BR	638.4 ± 209.2	598.2 ± 163.6	0.60
BRE	449.5 ± 245.4	555.7 ± 230.4	0.35
Citrate synthase			
BR	84.8 ± 17.1	60.34 ± 15.1	0.0012***
BRE	69.8 ± 41.4	60.07 ± 26.6	0.56

Activities are expressed as mU/mg of protein. Values are means ± SD. *; $P < 0.05$, **; $P < 0.01$, ***; $P < 0.001$.

Table 2. Gene expression (mRNA) of selected enzymes and growth/transcriptional factors of subjects undergoing 84 d bed rest with (BRE; n = 9) or without (BR; n = 12) flywheel resistance exercise.

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BR	52.82 ± 11.4	53.8 ± 22.7	0.89
BRE	50.52 ± 16.8	59.87 ± 18.2	0.27
Hexokinase-2			
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BR	69.01 ± 15.7	43.33 ± 8.5	< 0.00001***
BRE	63.17 ± 12.9	61.47 ± 13.6	0.078
SDH A			
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BRE	21.89 ± 5.3	19.82 ± 6.2	0.5
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SDH C			

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BR	0.82 ± 0.23	0.62 ± 0.25	0.031*
BRE	0.78 ± 0.19	0.51 ± 0.10	0.014*
VEGF			
BR	2.90 ± 0.61	2.15 ± 1.13	0.009**
BRE	3.13 ± 1.08	1.66 ± 0.53	< 0.0001***
Myostatin			
BR	0.22 ± 0.09	0.52 ± 0.18	< 0.0001***
BRE	0.22 ± 0.07	0.14 ± 0.04	0.011**

Gene expression levels are expressed as arbitrary units. Values are means ± SD. SDH A; succinate dehydrogenase a subunit, SDH B; succinate dehydrogenase b subunit, SDH C; succinate dehydrogenase c subunit, SDH D; succinate dehydrogenase d subunit, PGC-1α; peroxisome proliferator-activated receptor gamma coactivator-1, VEGF; vascular endothelial growth factor. *; $P < 0.05$, **; $P < 0.01$, ***; $P < 0.001$.

Table 3. Pearson's correlation coefficient (r) of relative changes from PRE to POST in enzyme activity of subjects undergoing 84 d bed rest with (BRE; n = 9) or without (BR; n = 12) flywheel resistance exercise.

	BR	BRE
GS vs. GPh	0.68*	0.55
GS vs. HK	0.77**	0.95**
GS vs. PFK-1	0.56	0.93**
GS vs. CS	0.57*	0.94**
GPh vs. HK	0.65*	0.59
GPh vs. PFK-1	0.54	0.67
GPh vs. CS	0.79**	0.62
HK vs. PFK-1	0.75*	0.99**
HK vs. CS	0.73**	0.99**
PFK-1 vs. CS	0.82**	0.99**

GS; glycogen synthase, GPh; glycogen phosphorylase, HK; hexokinase, PFK-1; phosphofructokinase, CS; citrate synthase. *; $P < 0.05$, **; $P < 0.01$.

Table 4. Discriminant analysis by Wilks' Lambda method applied to the non-dimensional ratio (INC = ([post_value] - [pre_value]) / [pre_value]). The right column shows the P

value for the difference in INC across groups (i.e. 84 d bed rest vs. 84 d bed rest with resistance exercise).

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GPh (activity)		0.47	0.46	0.34	0.23	0.22	0.006**
GPh (mRNA)			0.35	0.33	0.20	0.16	0.326
SDH B (mRNA)				0.23	0.22	0.18	0.008**
SDH D (mRNA)					0.18	0.15	0.854
HK-2 (mRNA)						0.12	0.046*

GPh; glycogen phosphorylase, SDH B; succinate dehydrogenase b subunit, SDH D; succinate dehydrogenase d subunit, HK-2; hexokinase 2. *; $P < 0.05$, **; $P < 0.01$, ***; $P < 0.001$.