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Effects of resistance exercise combined with essential amino acid supplementation and energy deficit on markers of skeletal muscle atrophy and regeneration during bed rest and active recovery

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Abstract

INTRODUCTION—Space flight and bed rest (BR) lead to muscle atrophy. This study assessed the effect of essential amino acid supplementation (EAA) and resistance training with decreased energy intake on molecular changes in skeletal muscle after 28d BR and 14d recovery.

METHODS—Thirty-one men (31–55yr) subjected to an 8±6% energy deficit were randomized to receive EAA without resistance training (AA, n=7), EAA 3 h after (RT, n=12), or 5 min before (AART, n=12) resistance training.

RESULTS—During BR, myostatin transcript levels increased 2-fold in the AA group. During recovery, IGF1 mRNA increased in all groups while Pax7, MyoD, myogenin and MRF4 transcripts increased in AA only (all p<0.05). MAFbx transcripts decreased 2-fold with AA and RT. Satellite cells did not change during BR or recovery.

DISCUSSION—This suggests that EAA alone is the least protective countermeasure to muscle loss, and several molecular mechanisms are proposed by which exercise attenuates muscle atrophy during bed rest with energy deficit.

Keywords

bed rest; resistance exercise; energy deficit; essential amino acid supplementation; myogenic factors

Introduction

Pronounced losses of skeletal muscle mass and strength are seen during space flight and in ground-based models such as bed rest 1–7. Exercise 3–8–11, essential amino acid (EAA) supplementation alone 12 or a combination 13 are countermeasures that alleviate, in part, the

loss of muscle mass and strength elicited by disuse (i.e. bed rest). Evidence indicates that hypogravity-induced skeletal muscle mass loss is amplified by anorexia 14·15. Our group has previously shown that essential amino acid supplementation combined with resistance exercise is not sufficient to fully maintain skeletal muscle mass during bed rest with energy deficit. However, exercise combined with amino acid supplementation 5 min before or 3 hr after exercise is more protective against muscle atrophy than essential amino acid supplementation alone 13. Specifically, mid-thigh muscle loss was less pronounced in participants subjected to exercise and amino acid supplementation (~4%) compared to those who received the amino acid supplement alone (~11%) 13.

Investigations using models of muscle disuse have yielded a large body of knowledge. In rodents subjected to hindlimb suspension 16 or space flight 17, the muscle specific ubiquitin ligases MAFbx and MuRF1 are up-regulated. Likewise, myostatin, a negative regulator of skeletal muscle that inhibits myofiber growth and differentiation as well as protein synthesis 18·19 is up-regulated in the hindlimb muscles of mice subjected to space flight 17·20 or hindlimb unloading 21. In humans, MAFbx transcript levels are elevated fourteen days after limb immobilization 22. A recent study documented increases in MAFbx and MuRF1 transcript levels as early as 48 hours after initiation of limb immobilization 23. Importantly, significant reductions in MAFbx, MuRF1 and myostatin transcript levels were achieved within 24 hours of initiation of rehabilitation 22. To date, the mechanisms by which the combination of essential amino acid supplementation and resistance training alleviates disuse muscle atrophy in humans are unknown.

In this study, we assessed the effects of essential amino acid supplementation alone or in combination with resistance training in the setting of energy deficit on markers of muscle atrophy and regeneration as well as satellite cell frequency in the *vastus lateralis* muscle of individuals subjected to a 28-day period of bed rest and a 14-day period of active recovery. We specifically investigated whether combined resistance exercise and essential AA supplementation would be more beneficial than essential AA supplementation alone in: 1) reducing skeletal muscle catabolic pathways while promoting anabolic or regeneration programs, and 2) expanding the muscle satellite cell pool. We also tested for associations between measures of muscle mass or strength and markers of muscle atrophy and regeneration as well as satellite cell frequency.

Methods

Ethical Approval

The study was approved by the Institutional Review Board at Tufts University and Tufts Medical Center in Boston, MA. Informed consent was obtained in writing from each participant prior to enrolling into the study.

Study Design

The study design has been reported elsewhere 13. Briefly, the 31 participants were healthy males (aged 31–55 years) with body mass indices ranging from 23–31 kg/m², with no contraindications to exercise and no dietary restrictions that would have prevented consumption of the study diet or the essential amino acid supplement. Participants reported to the Jean Mayer USDA HNRCA on day 0 and resided at the Metabolic Research Unit for the duration of the study (49 days). Each participant progressed through three phases: baseline (7 days), bed rest (28 days), and active recovery (14 days). During the baseline phase, participants acclimated to the site and were subjected to various tests. At the end of baseline and prior to bed rest, participants were randomized to one of the following countermeasure groups: essential amino acid supplementation alone (AA, n=7), resistance

training with AA supplementation provided 3 hours after exercise (RT, n=12), and resistance training with AA supplementation provided 5 minutes prior to exercise (AART, n=12).

Energy Intake

During the baseline period, each participant was provided with a weight maintenance diet which consisted of 15% protein, 54% carbohydrate, and 33% fat. During the bed rest and the active recovery phases, total energy intake was reduced by $8 \pm 6\%$ across all study groups while macronutrient intake composition was maintained 15.

Bed Rest

All participants underwent bed rest in the supine position for 28 days. Compliance was monitored by use of video cameras.

Essential Amino Acid Supplement

The supplement consisted of 15 g essential amino acids and 35 g sucrose (to improve palatability) dissolved in 500 ml water, and amounted to 200 kcal. The drink was given daily, 6 days/week during bed rest and active recovery.

Resistance Exercise Training

Participants exercised for 1 hour/day, 6 days/week. Exercise sessions were performed in the morning (from 9 am to 12 noon) and were staggered by one hour to accommodate up to three exercise sessions under individualized supervision. A total of 24 exercise sessions were performed during the 28-day bed rest phase. Participants were transferred from their bed to the exercise equipment by wheeled gurney and remained in a supine position. All exercises were performed in the horizontal position on a Shuttle Accel resistance training apparatus (Contemporary Design Company, Glacier, WA). Resistance was provided by elastic cords attached from the stationary frame to the sliding carriage. Resistance for each exercise was quantified as the number of elastic cords and the distance moved by the sliding carriage. A progressive, moderate- to high-intensity resistance training protocol was performed as a split routine with alternating days of lower and upper body exercises. The target exercise intensity was 70–80% of one-repetition maximum (1RM) estimated by the OMNI rating of perceived exercise (RPE) 10-category scale 24. Each session consisted of seven to eight exercises targeting major muscle groups and joint actions. Upper body exercises included pull-up, pull-over, triceps press, chest fly, shoulder press, biceps curl, upright row, and lateral arm raise. Lower body exercises included squats, single leg squats, diagonal jump, calf raise, single leg hip extension, leg curl, and single leg hip abduction. Exercises were choreographed to minimize positional and postural changes.

Active Recovery

Each participant performed 15–30 minutes of treadmill exercise at 60–85% of age-predicted maximum heart rate, 3 days/week. Intensity and duration of the treadmill exercise gradually increased over the 14 day period of recovery. In addition to treadmill exercise, subjects in the RT and AART groups underwent resistance training on alternate days using the Cybex Selectorized Equipment (Fresno, CA). Three sets of 8 repetitions were performed for each of the following 5 exercises: leg press, chest press, knee extension, knee flexion, and lat pull-down. Intensity increased over the 14-day recovery phase.

Outcome Measures

Functional measures and tissue samples were obtained at the end of baseline (prior to randomization), at the end of bed rest, and at the end of the active recovery phase. Functional measures are reported elsewhere 13.

Muscle Biopsies—Percutaneous needle biopsies were obtained from the *vastus lateralis* muscle of the non-dominant leg using a 5 mm Bergstrom needle with suction 25. Muscle tissues for quantification of transcripts were immediately frozen in liquid nitrogen. Muscle tissues for histochemical analysis were frozen in embedding medium (Tissue-Tek OCT, Miles Laboratories, Elkhart, IN) placed in liquid nitrogen cooled isopentane. These embedded samples were stored in liquid nitrogen.

Immunohistochemical Analysis—Sections (8µm thick) were fixed in paraformaldehyde. Sections were blocked in 20% goat serum, washed in phosphate buffered saline (PBS), and exposed overnight at 4°C to undiluted mouse anti-human Pax7 mAb (Developmental Hybridoma Bank, IA). Sections were washed in PBS, incubated in the dark at RT for 1 hr with an Alexa488 conjugated goat anti-mouse IgG (1:200, Cell Signaling, MA), washed in PBS, incubated in the dark at RT for 4 hrs with rabbit anti-human laminin antibody (1:200 in PBS, DAKO, Denmark), washed in PBS, incubated in the dark at RT for 1 hr with an Alexa594 conjugated goat anti-rabbit IgG (1:200, Cell Signaling), washed in PBS, incubated with the Hoescht dye 33342 (1:200 in PBS, Sigma Aldrich), washed in PBS and mounted with fluorescent mounting medium (DAKO). Slides were stored in the dark at -20°C until fluorochromes were activated by use of a fluorescent microscope. Fluorescence emitted by each of the three fluorochromes was sequentially captured. Pictures were merged as shown in Figure 1. Myonuclei were identified with Hoescht dye staining. Satellite cells were identified as Pax7⁺ nucleated cells residing within the basal lamina.

Total RNA Extraction and Transcript Quantification—Muscle samples (~ 30 mg) were homogenized using a polytron homogenizer (Tissue Tearor, BioSpec Products, Inc., Bartlesville, OK) in a mono-phase solution of phenol and guanidine thiocyanate (TRI-Reagent, Molecular Research Center, Cincinnati, OH). Total RNA was extracted with Ribopure (Ambion, Austin, TX). In some instances, following digestion of muscle tissues with proteinase K, total RNA was extracted with RNeasy (Qiagen, Valencia, CA). To ensure removal of genomic DNA contaminants, all samples were subjected to RNase-free DNase for on-column digestion (Qiagen). Total RNA was eluted in diethylpyrocarbonate-treated water. Nucleotide concentration was determined by UV spectrophotometry. Total RNA (1 µg) was converted to cDNA by the MMLV reverse transcriptase in the presence of random hexamers, dNTP mixture and RNase inhibitor (Taqman Reverse Transcription Reagents, Applied Biosystems, Foster City, CA).

First-strand cDNAs were amplified, and cDNA levels were quantified by real-time PCR. Amplifications were performed in an ABI PRISM 7700 Sequence Detector. Target cDNA levels were normalized to the levels of cDNA for S29, an internal control for which the expression is known not to vary in muscles subjected to exercise 26,27. The following primers and FAM-labeled probes to quantify myostatin (Hs00193363_m1), Pax7 (Hs00704053_s1), MyoD (Hs00159528_m1), myogenin (Hs00231167_m1), MRF4 (Hs00231165_m1) and S29 (Hs03004310_g1) were pre-designed by, and purchased from Applied Biosystems. The primer/probe sets to quantify MAFbx, MuRF1 and IGF1 cDNAs were designed by us using the software Primer Express v1.5, and purchased from Applied Biosystems. Sequences are as follows: MAFbx probe: FAM-5'-cct cag cag tta ctg caa-3'-MGB, MAFbx forward: 5'-aag cgc ttc ctg gat gag aa-3', MAFbx reverse: 5'-ttg gct gca aca tca tag ttc ag-3'; MuRF1 probe: FAM-5'-aca tct tcc agg ctg caa-3'-MGB, MuRF1 forward:

5'-tgc cgg aag tgt gcc aat-3', MuRF1 reverse: 5'-tcc gtg acg atc cat gat ca-3'; IGF1 probe: FAM-5'-caa gac cca gaa gga agt a-3'-MGB, IGF1 forward: 5'-agc gcc aca ccg aca tg-3', IGF1 reverse: 5'-ctt gtt tcc tgc act ccc tct ac-3'.

Statistical Analysis

Outcomes obtained from participants who completed the bed rest and recovery phases were analyzed using SPSS 17.0 for Windows (SPSS, Inc., Evanston, IL). Differences were considered statistically significant when two-tailed p values were ≤ 0.05 . Data are reported as mean and standard error (SE). Repeated measures analyses of variance (ANOVA) were used to assess time-by-group interactions in outcomes measured from end of baseline to end of bed rest, from end of bed rest to end of recovery, and from end of baseline to end of recovery. Tukey's post-hoc adjustments for multiple comparisons were performed. Pearson's coefficient of correlation was used to assess univariate associations among study outcomes. For this purpose we first run separate correlations for the resistance training groups (RT and AART) and the AA group. We then proceeded to run the correlations with all three groups combined. Given that results did not differ by either method, we present the data for the latter.

Results

Transcripts Encoding Atrophic Factors

Baseline myostatin ($p=0.16$), MAFbx ($p=0.38$) and MuRF1 ($p=0.17$) transcript levels were not statistically different among groups. After the intervention, myostatin transcript levels were increased 2-fold by the end of bed rest ($p=0.034$ for a time effect) in the AA group but were similar to baseline values by the end of recovery (Figure 2A). No time effects on myostatin transcript levels were observed in the RT and AART groups. MAFbx transcript levels were not affected by bed rest in any of the three groups but were decreased by 2-fold by end of recovery in the AA and RT groups ($p=0.048$ for a time effect; Figure 2B). No changes in MAFbx transcript levels were observed in the AART group. MuRF1 transcript levels did not change over the course of the study, regardless of the countermeasure (Figure 2C).

At baseline, myostatin transcript levels strongly correlated with those for MuRF1 ($r = 0.79$; $p<0.001$) and MAFbx ($r = 0.80$; $p<0.001$). At the end of bed rest, these associations remained significant: MAFbx ($r = 0.68$; $p<0.001$) and MuRF1 ($r = 0.60$; $p=0.001$). At the end of recovery, myostatin transcript levels correlated with those for MAFbx ($r = 0.53$; $p=0.007$), but not for MuRF1. MAFbx and MuRF1 transcript levels strongly correlated at end of baseline ($r = 0.90$; $p<0.001$), but less so at the end of bed rest ($r = 0.52$; $p=0.005$) and at the end of recovery ($r = 0.58$; $p=0.003$).

Transcripts Encoding Growth Factors and Myogenic Regulatory Factors

Baseline IGF1 ($p=0.73$), Pax7 ($p=0.09$), MyoD ($p=0.60$), myogenin ($p=0.10$) and MRF4 ($p=0.28$) transcript levels were not statistically different among groups. After the intervention, IGF1 transcript levels were elevated by end of recovery when compared with baseline in each of the three groups ($p=0.012$ for a time effect, Figure 3A). Transcript levels for the satellite cell marker Pax7 were already elevated after bed rest in the AART group (1.9 fold), but only after active recovery in the AA (1.9 fold) and RT (1.7 fold) groups (Figure 3B). In the AART group, Pax7 transcript levels did not further increase during active recovery. Significant time effect ($p<0.001$) and time-by-group interaction ($p=0.024$) were noted with respect to MyoD transcript levels (Figure 3C). Specifically, the AA group differed from the two exercising groups for which changes between RT and AART were not statistically different by post-hoc analysis ($p=0.55$). In the AA group, MyoD transcript levels

were increased by 2.0- and 2.6-fold at the end of bed rest and end of recovery when compared with baseline, respectively. In contrast, no increase was observed in the RT and AART groups. Myogenin transcript levels were elevated by 1.3 fold at end of recovery in the AA group ($p=0.042$ for a time effect; Figure 3D). No significant changes in myogenin transcript levels were noted in the RT and AART groups. MRF4 transcript levels also showed a significant time effect ($p=0.011$) driven by a 1.5-fold increase at the end of recovery in the AA group (Figure 3E).

At the end of baseline, IGF1 transcript levels were correlated with those for Pax7 ($r = 0.60$; $p<0.001$) and MyoD ($r = 0.66$; $p=0.001$). By the end of bed rest, IGF1 was correlated with Pax7 ($r=0.65$; $p<0.001$) and MyoD ($r=0.80$; $p<0.001$), but also with myogenin ($r=0.76$; $p<0.001$) and MRF4 ($r=0.52$; $p=0.013$). By the end of recovery, however, such associations were no longer observed. At the end of bed rest, there were strong correlations among the MRFs: MyoD and myogenin ($r=0.93$; $p<0.001$), MyoD and MRF4 ($r=0.72$; $p<0.001$); myogenin and MRF4 ($r=0.64$; $p=0.001$). At the end of recovery these associations remained: MyoD and myogenin ($r=0.88$; $p<0.001$), MyoD and MRF4 ($r=0.88$; $p<0.001$); myogenin and MRF4 ($r=0.78$; $p<0.001$). Myostatin transcript levels also correlated significantly with IGF1 transcript levels at baseline ($r=0.69$; $p<0.001$), end of bed rest ($r=0.66$; $p<0.001$) and end of recovery ($r=0.59$; $p=0.002$). Myostatin and MyoD were significantly correlated by the end of bed rest ($r=0.58$; $p=0.009$), but not at baseline or at end of recovery.

Satellite Cells and Myonuclei

Frequencies of satellite cells (expressed as percent of total nuclei) did not change significantly over the course of the study (Table 1). Myonuclei, measured as myonuclei per fiber, were not different among groups at baseline and did not change significantly among groups after bed rest. However, there was a trend for a gradual decrease in myonuclei over time in the AA group ($p=0.07$, for a time effect) as well as a significant time-by-group interaction ($p=0.002$) in myonuclei values between the end of recovery and baseline among groups (Table 1). This appears to be driven by a reduction in myonuclei in the AA group. The changes between the exercising groups (RT and AART) were not statistically different by post-hoc analysis ($p=0.87$). There were no significant correlations between satellite cell frequency expressed as percentage or with myonuclei measured at baseline, at the end of bed rest, or at the end of recovery with any of the transcripts measured at the same respective time points (data not shown).

Correlates of Muscle Mass and Transcript Levels in Skeletal Muscle

We previously reported that mid-thigh cross-sectional area and lower body strength declined more significantly during bed rest in the AA group than in the exercise training groups (RT or AART), and that the AA group demonstrated the least improvement in these parameters at the end of active recovery 13. Therefore, we tested for relationships between the previously reported measures of muscle mass and strength and transcript levels for markers of muscle atrophy, growth and regeneration. The only statistically significant correlations found between measures of muscle mass or strength and those of muscle transcripts at any of the three study time points (baseline, end of bed rest and end of recovery) were the following. At the end of bed rest, mid-thigh muscle area (ascertained by CT) was inversely correlated with transcript levels for myostatin ($r = -0.37$; $p=0.05$) and MuRF1 ($r = -0.55$; $p=0.003$) when all participants were included in the analysis.

Discussion

This study examined, for the first time, the adaptive molecular response of human skeletal muscle to disuse in the setting of restricted energy intake. We particularly aimed to identify

markers and molecular pathways of muscle atrophy or regeneration that would be differentially affected by countermeasures such as dietary manipulation alone or in combination with resistance exercise. We previously observed that the combination of essential amino acid supplementation and resistance training was superior to essential amino acid supplementation alone in minimizing the declines in mid-thigh muscle mass and strength. We now report that the combination of these countermeasures i) prevented the accumulation of transcripts that encode myostatin, a negative regulator of muscle mass, during bed rest but ii) did not require a strong myogenic program for an effective active recovery. We also report that IGF1 transcript levels were up-regulated during the active recovery period regardless of the countermeasure, suggesting that IGF1 is mobilized in atrophic conditions, whether mild or severe.

Myostatin, a negative regulator of skeletal muscle mass, inhibits muscle differentiation, protein synthesis and growth 18,19. Several studies of rodents subjected to muscle disuse have documented an increase in myostatin transcript levels. Myostatin transcripts are elevated in skeletal muscles of mice as early as one day after initiation of hindlimb unloading 21. Likewise, myostatin transcripts are elevated in muscles of rats after 17 days of space flight 20 and in those of mice 11 days into space flight, although the increase did not reach significance in the latter study 17. In our study, myostatin transcripts had accumulated by the end of bed rest in muscles of participants who received essential amino acids only, but not in those from participants who underwent resistance exercise in combination with essential amino acid supplementation. Furthermore, myostatin transcript levels at the end of bed rest correlated inversely with mid-thigh muscle area. These data suggest that i) myostatin contributes to the lower body muscle mass elicited by bed rest and ii) the protection conferred by resistance training relies, in part, on the suppression of myostatin gene expression. We acknowledge that myostatin gene deficiency does not prevent muscle disuse atrophy in mice 28 but also consider the possibility that myostatin may play a role in human muscle disuse atrophy, particularly in bed rest. In muscles of participants provided with essential amino acids alone, myostatin transcript levels were back to baseline levels by the end of the recovery period, implying that low myostatin gene expression is conducive for a strong recovery from muscle disuse atrophy.

The muscle specific ubiquitin ligase MAFbx has been identified as a factor that promotes muscle atrophy in several models of disuse 29. Relevant to our study is the upregulation of MAFbx in rodents subjected to hindlimb unloading 16 and space flight 17. We found that MAFbx was not up-regulated after bed rest but was decreased after active recovery in muscles of participants who received essential amino acids alone or 3 hours after exercise. In a previous study of limb immobilization in humans, MAFbx transcript levels were found to be elevated in muscles as early as 48 hours and as late as 14 days after initiation of disuse 22. The failure to detect an accumulation of MAFbx transcripts in our study may be best explained by the length of the bed rest period, i.e., 28 days. MAFbx gene expression may have been triggered in the early phase of bed rest, but it may have resolved before atrophied muscles reached a new homeostatic level. A similar interpretation may apply for the lack of accumulation of MuRF1 transcripts by the end of our bed rest period. It is noteworthy that MAFbx transcript levels decreased during active recovery in muscles of participants who received essential amino acids 3 hours after resistance exercise but not in those who received essential amino acids 5 min before resistance exercise, although these two countermeasures protected mid-thigh muscle mass and strength to a similar extent. This observation implies that MAFbx is not a major determinant of the protection conferred by the combination of these two countermeasures. Given that the induction of MAFbx transcript levels by exposure of C2C12 cells to dexamethasone is suppressed by addition of branched-chain amino acids 30, our observation suggests that the timing of essential amino acid supplementation is critical for suppression of MAFbx gene expression during the active

recovery period. Myostatin transcripts were strongly correlated with MuRF1 and MAFbx at baseline and at the end of bed rest, but after recovery, myostatin only correlated with MAFbx. This may be indicative of coordinated gene expression between myostatin and MAFbx in response to an atrophic stimulus (i.e. bed rest). Moderate doses of myostatin have previously been reported to lead to atrophy without concomitant increases in MAFbx and MuRF1 31, suggesting that myostatin may inhibit the mTOR pathway that affects predominantly protein synthesis rather than protein degradation 32. However, additional studies are needed to address this question.

IGF1 is a critical growth factor that promotes muscle hypertrophy. In the present study, the accumulation of IGF1 transcripts was marked in all participants after active recovery but not after bed rest. Earlier reports indicate that phosphorylation of Akt and FOXO by IGF1 results in reduced MAFbx gene transcription. Yet, in our study, the accumulation of IGF1 transcripts and the decline in MAFbx transcripts were concomitant only in muscles of participants who received essential amino acids alone or 3 hours after resistance training. Thus, our results suggest that MAFbx gene expression may be uncoupled from IGF1 activity in participants provided with essential amino acid supplementation 5 minutes before resistance training. Because IGF1 transcripts accumulated to a similar extent in the three countermeasure groups even though the groups differed by the degree of muscle mass loss and recovery, we conclude that IGF1 gene expression is a regenerative mechanism triggered by muscle atrophy regardless of its intensity, and that IGF1 was not a major determinant of the protection conferred by the countermeasures studied.

To further our understanding of the molecular pathways involved in the regeneration of muscle tissue during recovery and in the protection conferred by our countermeasures, we measured transcript levels for several myogenic factors. In muscles of participants who received essential amino acid supplementation only, MyoD transcripts were elevated by the end of bed rest and remained elevated by the end of active recovery. The accumulation of MyoD transcripts after active recovery is expected, because MyoD has been implicated in the regeneration of muscle elicited by exercise 33-34. In contrast, the accumulation of MyoD transcripts after bed rest is paradoxical and may represent an inefficient attempt to regenerate muscle tissue at time of intense muscle proteolysis 13. In these participants who received essential amino acids only, the levels of transcripts that encode other myogenic factors such as myogenin and MRF4 were elevated by the end of active recovery, but not at the end of bed rest. For each of these myogenic factors, no changes in transcript levels were observed throughout the study in muscles of participants provided with essential amino acid supplementation before or after resistance exercise. We conclude that resistance training combined with essential amino acids protects skeletal muscle from disuse atrophy to the extent that there is no need for a major mobilization of myogenic factors during bed rest and active recovery. Interestingly, we found IGF1 and MyoD transcripts to be significantly correlated with myostatin expression after 28 days of bed rest. Previous studies have shown that myostatin levels are inversely related to MyoD expression 18-35. In a recent study investigating underfeeding in sheep, there were immediate decreases in levels of IGF1, myostatin and MRFs followed by increases in myostatin and MyoD as soon as one week of underfeeding. In contrast, long term underfeeding (20 weeks) led to increased IGF1 and MRFs transcript levels 36, suggesting that the association between myostatin and MRFs may be time dependent.

By integrating mature post-mitotic fibers, satellite cells participate in the maintenance of muscle mass in response to various stimuli [for review, see 37]. In our study, we use the transcription factor Pax7 as a marker of mobilization of the satellite cell pool. By the end of recovery, Pax7 transcript levels were elevated in the muscles of participants from each study group. When essential amino acid supplementation was provided 5 min before resistance

exercise training, however, Pax7 transcript levels were already elevated by the end of bed rest. As Pax7 expression tends to be associated with self-renewal and quiescence of the satellite cell population, an increase in expression may be indicative of the maintenance of the existing satellite cell pool 38. Thus, a constant satellite cell number throughout the study may be the result of parallel satellite cell loss and replenishment. Despite evidence of satellite cell activation, we found no changes in satellite cell frequency after bed rest or active recovery. We did note a trend for a gradual decrease in myonuclei over time in the AA group. In contrast, the combination of amino acid supplementation with resistance exercise may have contributed to maintenance of myonuclear levels after bed rest in the RT and AART groups. A previous study reported a loss of myofiber nuclei in the soleus muscles of rats that underwent atrophy due to 10 days of spaceflight 39. However, the myonuclear domain sizes were identical between control and flight animals, suggesting that myonuclei decreased in numbers as the fibers atrophied to maintain a relatively constant size of the myonuclear domain. The potential of this response in the presence of the countermeasures we studied will need to be investigated further.

In conclusion, muscle atrophy imposed by 28 days of bed rest and muscle regeneration associated with active recovery are complex processes characterized by changes in the accumulation of transcripts that encode anabolic and catabolic factors and myogenic regulatory factors. These changes are discrete, appear coordinated, and are stage specific. Muscle atrophy was greatest among participants who received essential amino acids only, and it was characterized by a marked accumulation of myostatin transcripts. Active recovery was associated with elevated levels of transcripts that encode an array of myogenic and anabolic factors. The combination of resistance training and essential amino acid supplementation attenuated muscle disuse atrophy due to bed rest, but it did not trigger a strong myogenic program during active recovery.

Further studies are needed to fully understand the complex molecular regulators of muscle atrophy and regeneration in ground-based models of disuse such as bed rest, and to identify how these pathways are affected by resistance training and essential amino acid supplementation. Given that the objective of this study was to mimic the anorexia of space flight, our study design called for restricted dietary intake and did not include study groups that received adequate energy intake or bed rest with or without countermeasures. The findings of this study are novel and warrant further studies to investigate several modalities of countermeasures that may vary by the type and timing of essential amino acids supplemented as well as by the intensity and duration of exercise, particularly in the context of adequate energy intake.

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Abbreviations

BR	bed rest
EAA	essential amino acid
ANOVA	analysis of variance
IGF1	insulin-like growth factor 1
MAFbx	muscle atrophy F-box
MuRF1	Muscle Ring Finger 1
Pax7	paired box protein 7
MRF	myogenic regulatory factor
CT	computerized tomography
Countermeasure groups:	essential amino acid supplementation alone
AA	
RT	resistance training with AA supplementation provided 3 hours after exercise
AART	resistance training with AA supplementation provided 5 minutes prior to exercise

References

1. Bloomfield SA. Changes in musculoskeletal structure and function with prolonged bed rest. *Med Sci Sports Exerc.* 1997; 29:197–206. [PubMed: 9044223]
2. Edgerton VR, Zhou M-Y, Ohira Y, Klitgaard H, Jiang B, Bell G, Harris B, Saltin B, Gollnick PD, Roy RR, Day MK, Greenisen M. Human fiber size and enzymatic properties after 5 and 11 days of spaceflight. *J Appl Physiol.* 1995; 78:1733–1739. [PubMed: 7649906]
3. Ferrando AA, Lane HW, Stuart CA, Davis-Street J, Wolfe RR. Prolonged bed rest decreases skeletal muscle and whole body protein synthesis. *Am J Physiol.* 1996; 270:E627–E633. [PubMed: 8928769]
4. Greenleaf JE, Kozlowski S. Physiological consequences of reduced physical activity during bed rest. *Exerc Sport Sci Rev.* 1982; 10:84–119. [PubMed: 6749519]
5. Hikida RS, Gollnick PD, Dudley GA, Convertino VA, Buchanan P. Structural and metabolic characteristics of human skeletal muscle following 30 days of simulated microgravity. *Aviat Space Environ Med.* 1989; 60:664–670. [PubMed: 2764850]
6. LeBlanc AD, Schneider VS, Evans HJ, Pientok C, Rowe R, Spector E. Regional changes in muscle mass following 17 weeks of bed rest. *J Appl Physiol.* 1992; 73:2172–2178. [PubMed: 1474100]
7. Suzuki Y, Murakami T, Kawakubo K, Haruna Y, Takenaka K, Goto S, Makita Y, Ikawa S, Gunji A. Regional changes in muscle mass and strength following 20 days of bed rest, and the effects on orthostatic tolerance capacity in young subjects. *J Gravit Physiol.* 1994; 1:P57–P58. [PubMed: 11538763]
8. Akima H, Kubo K, Imai M, Kanehisa H, Suzuki Y, Gunji A, Fukunaga T. Inactivity and muscle: effect of resistance training during bed rest on muscle size in the lower limb. *Acta Physiol Scand.* 2001; 172:269–278. [PubMed: 11531648]
9. Akima H, Ushiyama J, Kubo J, Tonosaki S, Itoh M, Kawakami Y, Fukuoka H, Kanehisa H, Fukunaga T. Resistance training during unweighting maintains muscle size and function in human calf. *Med Sci Sports Exerc.* 2003; 35:655–662. [PubMed: 12673150]
10. Bamman MM, Clarke MS, Feeback DL, Talmadge RJ, Stevens BR, Lieberman SA, Greenisen MC. Impact of resistance exercise during bed rest on skeletal muscle sarcopenia and myosin isoform distribution. *J Appl Physiol.* 1998; 84:157–163. [PubMed: 9451630]

11. Kawakami Y, Akima H, Kubo K, Muraoka Y, Hasegawa H, Kouzaki M, Imai M, Suzuki Y, Gunji A, Kanehisa H, Fukunaga T. Changes in muscle size, architecture, and neural activation after 20 days of bed rest with and without resistance exercise. *Eur J Appl Physiol.* 2001; 84:7–12. [PubMed: 11394257]
12. Paddon-Jones D, Sheffield-Moore M, Urban RJ, Sanford AP, Aarsland A, Wolfe RR, Ferrando AA. Essential amino acid and carbohydrate supplementation ameliorates muscle protein loss in humans during 28 days bedrest. *J Clin Endocrinol Metab.* 2004; 89:4351–4358. [PubMed: 15356032]
13. Brooks N, Cloutier GJ, Cadena SM, Layne JE, Nelsen CA, Freed AM, Roubenoff R, Castaneda-Sceppa C. Resistance training and timed essential amino acids protect against the loss of muscle mass and strength during 28 days of bed rest and energy deficit. *J Appl Physiol.* 2008; 105:241–248. [PubMed: 18483167]
14. Stein TP. Nutrition and muscle loss in humans during spaceflight. *Adv Space Biol Med.* 1999; 7:49–97. [PubMed: 10660773]
15. Stein TP, Leskiw MJ, Schluter MD, Hoyt RW, Lane HW, Gretebeck RE, LeBlanc AD. Energy expenditure and balance during spaceflight on the space shuttle. *Am J Physiol.* 1999; 276:R1739–R1748. [PubMed: 10362755]
16. Dupont-Versteegden EE, Fluckey JD, Knox M, Gaddy D, Peterson CA. Effect of flywheel-based resistance exercise on processes contributing to muscle atrophy during unloading in adult rats. *J Appl Physiol.* 2006; 101:202–212. [PubMed: 16601304]
17. Allen DL, Bandstra ER, Harrison BC, Thorng S, Stodieck LS, Kostenuik PJ, Morony S, Lacey DL, Hammond TG, Leinwand LL, Argraves WS, Bateman TA, Barth JL. Effects of spaceflight on murine skeletal muscle gene expression. *J Appl Physiol.* 2008
18. Langley B, Thomas M, Bishop A, Sharma M, Gilmour S, Kambadur R. Myostatin inhibits myoblast differentiation by down-regulating MyoD expression. *J Biol Chem.* 2002; 277:49831–49840. [PubMed: 12244043]
19. Zimmers TA, Davies MV, Koniaris LG, Haynes P, Esquela AF, Tomkinson KN, McPherron AC, Wolfman NM, Lee SJ. Induction of cachexia in mice by systemically administered myostatin. *Science.* 2002; 296:1486–1488. [PubMed: 12029139]
20. Lalani R, Bhasin S, Byhower F, Tarnuzzer R, Grant M, Shen R, Asa S, Ezzat S, Gonzalez-Cadavid NF. Myostatin and insulin-like growth factor-I and -II expression in the muscle of rats exposed to the microgravity environment of the NeuroLab space shuttle flight. *J Endocrinol.* 2000; 167:417–428. [PubMed: 11115768]
21. Carlson CJ, Booth FW, Gordon SE. Skeletal muscle myostatin mRNA expression is fiber-type specific and increases during hindlimb unloading. *Am J Physiol.* 1999; 277:R601–R606. [PubMed: 10444569]
22. Jones SW, Hill RJ, Krasney PA, O’Conner B, Peirce N, Greenhaff PL. Disuse atrophy and exercise rehabilitation in humans profoundly affects the expression of genes associated with the regulation of skeletal muscle mass. *FASEB J.* 2004; 18:1025–1027. [PubMed: 15084522]
23. Abadi A, Glover EI, Isfort RJ, Raha S, Safdar A, Yasuda N, Kaczor JJ, Melov S, Hubbard A, Qu X, Phillips SM, Tarnopolsky M. Limb immobilization induces a coordinate down-regulation of mitochondrial and other metabolic pathways in men and women. *PLoS One.* 2009; 4:e6518. [PubMed: 19654872]
24. Robertson RJ, Goss FL, Rutkowski J, Lenz B, Dixon C, Timmer J, Frazee K, Dube J, Andreacci J. Concurrent validation of the OMNI perceived exertion scale for resistance exercise. *Med Sci Sports Exerc.* 2003; 35:333–341. [PubMed: 12569225]
25. Evans WJ, Phinney SD, Young VR. Suction applied to a muscle biopsy maximizes sample size. *Med Sci Sports Exerc.* 1982; 14:101–102. [PubMed: 7070249]
26. Schjerling P. The importance of internal controls in mRNA quantification. *J Appl Physiol.* 2001; 90:401–402. [PubMed: 11189056]
27. Kraniou Y, Cameron-Smith D, Misso M, Collier G, Hargreaves M. Effects of exercise on GLUT-4 and glycogenin gene expression in human skeletal muscle. *J Appl Physiol.* 2000; 88:794–796. [PubMed: 10658052]

28. McMahon CD, Popovic L, Oldham JM, Jeanplong F, Smith HK, Kambadur R, Sharma M, Maxwell L, Bass JJ. Myostatin-deficient mice lose more skeletal muscle mass than wild-type controls during hindlimb suspension. *Am J Physiol Endocrinol Metab.* 2003; 285:E82–E87. [PubMed: 12618358]
29. Bodine SC, Latres E, Baumhueter S, Lai VK, Nunez L, Clarke BA, Poueymirou WT, Panaro FJ, Na E, Dharmarajan K, Pan ZQ, Valenzuela DM, DeChiara TM, Stitt TN, Yancopoulos GD, Glass DJ. Identification of ubiquitin ligases required for skeletal muscle atrophy. *Science.* 2001; 294:1704–1708. [PubMed: 11679633]
30. Herningtyas EH, Okimura Y, Handayaningsih AE, Yamamoto D, Maki T, Iida K, Takahashi Y, Kaji H, Chihara K. Branched-chain amino acids and arginine suppress MaFbx/atrogen-1 mRNA expression via mTOR pathway in C2C12 cell line. *Biochim Biophys Acta.* 2008; 1780:1115–1120. [PubMed: 18616983]
31. Amirouche A, Durieux AC, Banzet S, Koulmann N, Bonnefoy R, Mouret C, Bigard X, Peinnequin A, Freyssen D. Down-regulation of Akt/mammalian target of rapamycin signaling pathway in response to myostatin overexpression in skeletal muscle. *Endocrinology.* 2009; 150:E286–E294.
32. Welle S, Bhatt K, Pinkert CA. Myofibrillar protein synthesis in myostatin-deficient mice. *Am J Physiol Endocrinol Metab.* 2006; 290:409–415.
33. Okada A, Ono Y, Nagatomi R, Kishimoto KN, Itoi E. Decreased muscle atrophy F-box (MAFbx) expression in regenerating muscle after muscle-damaging exercise. *Muscle Nerve.* 2008; 38:1246–1253. [PubMed: 18785187]
34. Harber MP, Crane JD, Dickinson JM, Jemiolo B, Raue U, Trappe TA, Trappe SW. Protein synthesis and the expression of growth-related genes are altered by running in human vastus lateralis and soleus muscles. *Am J Physiol.* 2009; 296:R708–R714.
35. Hennebry A, Berry C, Siriatt V, O'Callaghan P, Chau L, Watson T, Sharma M, Kambadur R. Myostatin regulates fiber-type composition of skeletal muscle by regulating MEF2 and MyoD gene expression. *Am J Physiol Cell Physiol.* 2009; 296:C525–C534. [PubMed: 19129464]
36. Jeanplong F, Bass JJ, Smith HK, Kirk SP, Kambadur R, Sharma M, Oldham JM. Prolonged underfeeding of sheep increases myostatin and myogenic regulatory factor Myf-5 in skeletal muscle while IGF-I and myogenin are repressed. *J Endocrinol.* 2003; 176:425–437. [PubMed: 12630927]
37. Kadi F, Charifi N, Denis C, Lexell J, Andersen JL, Schjerling P, Olsen S, Kjaer M. The behaviour of satellite cells in response to exercise: what have we learned from human studies? *Pflugers Arch.* 2005; 451:319–327. [PubMed: 16091958]
38. Olguin HC, Olwin BB. Pax-7 up-regulation inhibits myogenesis and cell cycle progression in satellite cells: a potential mechanism for self-renewal. *Dev Biol.* 2004; 275:375–388. [PubMed: 15501225]
39. Hikida RS, Van Nostran S, Murray JD, Staron RS, Gordon SE, Kraemer WJ. Myonuclear loss in atrophied soleus muscle fibers. *Anat Rec.* 1997; 247:350–354. [PubMed: 9066912]

Figure 1. Satellite cells are distinguished from myonuclei using co-staining for Pax7, laminin and DNA

Panels are images of a representative cross-section of skeletal muscle tissue stained for: Pax7 (A), myonuclei with Hoescht 33342 (B), and laminin (C). The merge image is presented in panel D. Satellite cells are identified as Pax7 positive cells stained with Hoescht 33342, and located within the basal lamina as revealed by laminin.

Figure 2. Resistance training prevents the accumulation of myostatin transcripts induced by bed rest

Transcript levels for myostatin (A), MAFbx (B) and MuRF1 (C) are reported as mean $\pm$ SE. Open bars represent baseline values, closed bars represent values after 28 days of bed rest and gray bars represent values after 14 days of recovery. Participants were randomized to one of three groups: amino acid supplement only: AA; resistance training with amino acid supplement provided 3 hours after exercise: RT; or resistance training with amino acid supplement provided 5 minutes before exercise: AART. Time-by-group interactions between end of bed rest and baseline, end of recovery and end of bed rest, and end of recovery and baseline were tested by repeated measures ANOVA. * Indicates p values < 0.05 for a time effect.

Figure 3. Active recovery leads to increases in myogenic transcript levels in muscles of participants provided with essential amino acids only

Transcript levels for IGF1 (A), Pax7 (B), MyoD (C), myogenin (D) and MRF4 (E) are reported as mean $\pm$ SE. Open bars represent baseline values, closed bars represent values after 28 days of bed rest and gray bars represent values after 14 days of active recovery. Participants were randomized to one of three groups: amino acid supplement only (AA), resistance training with AA provided 3 hr after exercise (RT) or resistance training with AA provided 5 min before exercise (AART). Time-by-group interactions between end of bed rest and baseline, end of recovery and end of bed rest, and end of recovery and baseline were tested by repeated measures ANOVA.

* Indicates p values < 0.05 for a time effect. \$ Indicates p values < 0.03 for a time-by-group interaction.

Table 1

Effect of essential amino acid supplementation alone or in combination with resistance training on satellite cells and myonuclei.

	Baseline	End Bed Rest	End Recovery
AA			
Satellite cells (% total nuclei)	3.00 ± 0.43	3.20 ± 0.46	3.00 ± 0.43
Myonuclei/fiber	3.30 ± 0.22	2.91 ± 0.15	2.62 ± 0.11 *
RT			
Satellite cells (% total nuclei)	3.33 ± 0.30	2.80 ± 0.60	2.82 ± 0.44
Myonuclei/fiber	2.67 ± 0.16	2.81 ± 0.12	2.71 ± 0.17 *
AART			
Satellite cells (% total nuclei)	3.62 ± 0.40	2.48 ± 0.40	3.44 ± 0.57
Myonuclei/fiber	2.60 ± 0.16	2.63 ± 0.12	2.51 ± 0.17 *

Data are mean ± SE. AA: essential amino acid supplementation alone (n=7), RT: resistance exercise training with AA supplement provided 3 hr after exercise (n=11), and AART: resistance exercise training with AA supplement provided 5 min before exercise (n=11). Time-by-group interactions between end of bed rest and baseline, end of recovery and end of bed rest, and end of recovery and baseline were tested by repeated measures ANOVA.

* P=0.002 for a time-by-group interaction between end of recovery and baseline.