Age-related differences in lean mass, protein synthesis and skeletal muscle markers of proteolysis after bed rest and exercise rehabilitation

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Key points

- Five days of bed rest resulted in a reduction in leg lean mass and strength in older adults.
- After bed rest, older (but not younger) adults had reduced amino acid-induced anabolic sensitivity (blunted muscle protein synthesis; MPS) and enhanced markers associated with the ubiquitin proteasome and autophagy–lysosomal systems (increase in molecular markers related to muscle proteolysis).
- Younger adults did not lose leg lean mass (via DXA) after 5 days of bed rest despite blunted amino acid-induced mTORC1 signalling and increased skeletal muscle REDD1, REDD2 and MURF1 mRNA expression.
- Exercise rehabilitation restored bed rest-induced deficits in lean mass, strength, nutrient-induced protein anabolism (protein synthesis and mTORC1 signalling) and select muscle proteolytic markers in older adults.

Abstract Bed rest-induced muscle loss and impaired muscle recovery may contribute to age-related sarcopenia. It is unknown if there are age-related differences in muscle mass and muscle anabolic and catabolic responses to bed rest. A secondary objective was to determine if rehabilitation could reverse bed rest responses. Nine older and fourteen young adults participated in a 5-day bed rest challenge (BED REST). This was followed by 8 weeks of high intensity resistance exercise (REHAB). Leg lean mass (via dual-energy X-ray absorptiometry; DXA) and strength were determined. Muscle biopsies were collected during a constant stable isotope infusion in the post-absorptive state and after essential amino acid (EAA) ingestion on three occasions: before (PRE), after bed rest and after rehabilitation. Samples were assessed for protein synthesis, mTORC1 signalling, REDD1/2 expression and molecular markers related to muscle proteolysis (MURF1, MAFBX, AMPKα, LC3II/I, Beclin1). We found that leg lean mass and strength decreased in older but not younger adults after bedrest ($P < 0.05$) and was restored after rehabilitation. EAA-induced mTORC1 signalling and protein synthesis increased before bed rest in both age groups ($P < 0.05$). Although both groups had blunted mTORC1 signalling, increased REDD2 and MURF1 mRNA after bedrest, only older adults had reduced EAA-induced protein synthesis rates and increased MAFBX mRNA, p-AMPKα and the LC3II/I ratio ($P < 0.05$). We conclude that older adults are more susceptible than young persons to muscle loss after short-term bed rest. This may be partially explained by a combined suppression of protein synthesis and a marginal increase in proteolytic markers. Finally, rehabilitation restored bed rest-induced deficits in lean mass and strength in older adults.

Abbreviations   4EBP1, 4E binding protein 1; AMPKα, 5′ AMP activated protein kinase alpha; BED REST, 5 days of bed rest; DXA, dual-energy X-ray absorptiometry; EAA, essential amino acids; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LC3, microtubule associated protein 1 light chain 3; MPS, muscle protein synthesis; mTORC1, mechanistic target of rapamycin complex 1; MAFBX, muscle atrophy F-box; MURF1, muscle-specific RING finger 1; PRE, before bed rest; RPE, rating of perceived exertion; REHAB, 8 weeks of exercise rehabilitation; rpS6, ribosomal protein S6; REDD1 and 2, regulated in development and DNA damage 1 and 2; S6K1, S6 kinase 1.

Introduction

Deterioration of lower extremity skeletal muscle function (decreased lean tissue and strength) in the young and older adults is a frequent consequence of reduced physical activity such as during bed rest (Kortebein *et al.* 2007; Drummond *et al.* 2012; Dirks *et al.* 2014). This poor outcome is especially evident for many older adults who experience repeated episodes of low levels of ambulation during acute hospitalizations and recovery from injuries, illnesses or during the course of a disease (Fisher *et al.* 2011,2013). Furthermore, older adults exhibit poor muscle recovery following disuse-related muscle loss (Suetta *et al.* 2009,2013). Therefore, it is believed that inactivity-induced muscle loss coupled with incomplete muscle recovery contribute to age-related muscle sarcopenia.

Recently, we (Drummond *et al.* 2012) and others (Kortebein *et al.* 2007; Breen *et al.* 2013; Deutz *et al.* 2013) have shown that reduced physical activity or 7–10 days bed rest in older adults decreased lean tissue and compromised the anabolic potency to feeding/essential amino acids, e.g. blunted muscle protein synthesis (MPS) and mechanistic target of rapamycin (mTORC1) signalling, a major cell signalling pathway that regulates protein synthesis. These data are consistent with other physical inactivity (e.g. immobilization) and bed rest studies (7–14 days) in young adults (Ferrando *et al.* 1996; Biolo *et al.* 2004; Glover *et al.* 2008; Wall *et al.* 2013*b*) suggesting that decreased fasted and fed muscle protein synthesis rates are key aspects associated with physical inactivity-induced muscle loss. Muscle catabolic signalling pathways (e.g. ubiquitin proteosomal system, autophagy) and inhibitors of mTORC1 (REDD1 and REDD2) are far less described with bed rest in older adults. Recent cast immobilization studies in young and older adults show increased expression of skeletal muscle E3 protein ligases, MAFBX and MURF1, and markers of autophagy (LC3II/I) (Jones *et al.* 2004; Suetta *et al.* 2012; Dirks *et al.* 2014). Similarly, studies in rodents have shown that a pair of mTORC1 repressors, regulated in development and DNA damage (REDD1 and REDD2), are also increased with immobilization and are inversely correlated with mTORC1 signalling and protein synthesis (Kelleher *et al.* 2013,2015; Gordon *et al.* 2015). Examining indices of muscle protein breakdown and repressors of mTORC1 signalling would provide new insight into potential age-related differences in muscle loss with bed rest.

To date, there has not been a side-by-side comparison of the influence of bed rest on skeletal muscle function as a result of age. It is generally accepted that there is a higher rate of muscle loss in older adults with bed rest; yet with lower limb immobilization studies the opposite appears to occur (Suetta *et al.* 2009,2012; Wall *et al.* 2015). First, these assumptions with bed rest have been derived from examples of independent studies in young (LeBlanc *et al.* 1992; Ferrando *et al.* 1996; Trappe *et al.* 2008) and older (Kortebein *et al.* 2007; Drummond *et al.* 2012; Deutz *et al.* 2013) adults but unfortunately these studies have not used comparable durations of bed rest between the age groups. Secondly, none have examined the impact of less than 7 days of bed rest on older adult skeletal muscle. This is important because muscle loss in the older adult is predicted to be most rapid during the early stages of inactivity (Wall *et al.* 2013*a,b*).

As a secondary objective, we were also interested in determining if a rehabilitation period, defined as 8 weeks of high-intensity eccentric exercise and BCAA-enriched protein supplementation, could reverse bed rest-induced changes in lean mass, protein anabolism and skeletal muscle catabolic signalling. Our motivation was based on data supporting that older adults have impaired muscle mass recovery after immobilization (Suetta *et al.* 2009,2013). Therefore, we hypothesized that bed rest would result in an age-dependent decline in skeletal muscle mass that would be related to nutrient-induced muscle protein synthesis (and mTORC1 signalling) and surrogate markers of muscle protein breakdown (MURF1, MAFBX, AMPKα, LC3II, Beclin1). We also predicted that rehabilitation after bed rest in young and older adults would reverse bed-rest-induced changes in muscle structure and function and dysregulation of protein anabolism and protein catabolic signalling.

Methods

Subjects

We recruited 23 healthy community dwelling subjects from the Salt Lake City area in two age groups of

18–35 and 60–75 years and with a BMI <30 kg m^{-2} into this study: 14 young (7 male, 7 female) and 9 older adults (2 male, 7 female). Subjects were recruited through campus and community flyers, radio announcements, health fairs, ClinicalTrials.org and word-of-mouth. Subjects were screened through personal interview, physician evaluation and a blood panel to meet an extensive set of inclusion/exclusion criteria. The exclusion list contained various physical, mental, muscle or metabolic dysfunctions that may impact on protein metabolism or contraindicated for bed rest. These included uncontrolled hypertension, diabetes, HIV, hepatitis B and C, hyper/hypothyroidism, cardiovascular, kidney, respiratory, vascular and liver disease, history of DVT, neurological disorders, recent cancer treatment (within 1 year of enrolling), osteopenia, depression, and alcohol substance abuse. Young female subjects were not pregnant at the time of enrollment (as assessed with HCG) while some young females were using contraceptives during the length of the study ($n = 4$). Importantly, during the metabolic studies (see below), young women were studied during the follicular phase of their menstrual cycle. Some older adult subjects were on hypertensive medications ($n = 4$) or statins ($n = 3$) but refrained from use during the bed rest portion of this study. All subjects were moderately physically active defined as recreational walks, hikes, bike rides and/or participation in <2 sessions of aerobic or resistive-type activities/week. Subjects were not currently engaged in structured lower body strength resistance training for 6 months prior to enrolling in this study. Of the 14 young subjects that underwent bed rest, 7 young subjects were randomly assigned (restricted randomization in blocks of 4–1:1 ratio) to continue onto the rehabilitation phase (REHAB). The remaining young subjects ($n = 7$) were assigned to a separate intervention not included in this study. Therefore, the rehabilitation phase only contained data from seven young (3 female, 4 male, 22 ± 1 years, 23 ± 1 kg m^{-2} BMI) and nine older (7 female, 2 male, 66 ± 1 years, 25 ± 1 kg m^{-2} BMI) adult subjects. One of the females that moved onto the rehabilitation intervention was using contraceptives. All subjects read and signed the informed consent form. The study took place at the University of Utah (January 2012–December 2014) and was approved by the University of Utah Institutional Review Board, and conformed to the standards set by the *Declaration of Helsinki*.

Experimental design

Study events, timeline and metabolic study can be found in Fig. 1. In general, body composition and lower extremity strength were assessed before bed rest (PRE), after bed rest (BED REST) and after 8 weeks of exercise rehabilitation (REHAB). Bed rest consisted of a 5-day, 4-night (Monday - Friday) physical inactivity experiment

within the clinical research unit. REHAB encompassed 8 weeks of high intensity eccentric resistance exercise ($3\times$ per week) of both legs coupled with post-exercise BCAA-enriched protein supplementation. A metabolic study was conducted at PRE (bed rest day 1) and repeated after bed rest (day 5) and after REHAB for the purposes of capturing key physiological measurements associated with protein metabolism and regulation of muscle mass in young and older adults.

Tissue composition and strength testing

Two weeks before bed rest, and after an overnight fast, lean tissue was determined using a dual-energy X-ray absorptiometry (DXA) scan (Berg *et al.* 1993) by a trained technician at the University of Utah's Centre for Clinical and Translational Sciences, Clinical Research Unit. Subjects also underwent knee extension strength testing determined via a maximum voluntary isometric contraction developed by the knee extensors (at a 60 deg angle) on a KinCom dynamometer (Chattanooga Inc. Hixon, TN, USA) as described previously (Marcus *et al.* 2012). Each leg was tested individually and data were reported as peak torque (N·m) from the average of both legs. Strength testing was monitored by a research assistant. Tissue composition and isometric strength testing were repeated after BED REST and after REHAB.

Habitual dietary and physical activity assessment

Approximately 2 weeks prior to bed rest, subjects were given specific instruction to record food intake over 5 days (including one weekend day). Caloric intake was later calculated using the Food Processor Nutrition Analysis software (Salem, OR, USA). At the same time, subjects were provided with a step activity monitor (Orthocare Innovations, Mountain Lake Terrace, WA USA) to measure daily habitual physical activity.

Bed rest dietary control

The night before entering the Clinical Research Unit, subjects were given a pre-packaged dinner to consume consisting of a caloric and macronutrient density based on the individuals' body weight. Total caloric intake during bed rest was pre-determined by the research dietician using the Harris–Benedict equation adjusted for no physical activity. Additionally, subjects consumed a diet that provided a daily protein amount of ~ 1.0 g protein kg^{-1} day^{-1} protein intake. Subjects were also provided water *ad libitum* throughout the 5 days of bed rest.

Metabolic study

The metabolic study, conducted before bed rest (Day 1), after bed rest (Day 5) and after exercise rehabilitation,

was similar in design to our previous bed rest study (Drummond *et al.* 2012). After an overnight fast, L-[ring-^{13}C$_6$] phenylalanine was infused (priming dose: 2 μmol kg^{-1}, infusion rate: 0.05 μmol kg^{-1} min^{-1}) in an antecubital vein for 7 h. After applying 1% lidocaine and using aseptic technique, two muscle biopsies were sampled from the vastus lateralis using a modified Bergström needle approach with manual suction (Evans *et al.* 1982). Each biopsy was separated by 2 h for purposes of measuring fasted muscle protein synthesis rates. In a separate incision proximal to previous skin incision site, two additional muscle biopsies were taken 1 h and 3 h after ingestion of a L-[ring-^{13}C$_6$] phenylalanine-enriched (7.7%) essential amino acid (EAA) drink (~12 g; 2.5 g Leu). All muscle tissue was immediately washed with cold saline and dissected of visible non-muscle tissue, flash-frozen in liquid nitrogen, and stored at −80°C for later analysis.

5-day bed rest

After the completion of the first metabolic study, subjects adhered to five continuous days of bed rest. Our bed rest model was designed to mimic a traditional inpatient hospital stay and reflect the level of muscle unloading, rest and recuperation that occurs in older adults following an acute illness, injury or infection (Fisher *et al.* 2010). Subjects spent a majority of their time in bed and were allowed to adjust hospital bed head height for reading, eating and watching television but otherwise were instructed to lay the bed flat for sleeping. Bathing and hygiene activities were performed at the sink in a wheel chair. Subjects also accessed the bathroom using a wheel chair. Adherence to bed rest was monitored by nursing staff 24 h a day. In order to reduce discomfort and risk of deep venous thrombosis during bed rest, subjects were encouraged to change horizontal position (i.e. roll to side) and to bend their knees periodically. As part of standard of care for hospitalized/bed-ridden patients, subjects were treated with intermittent serial compression devices and were required to wear compression stockings on their lower limbs, and were provided with daily passive range of motion (by a physical therapist or physical therapy student) to the major lower extremity joints,. Additionally, blood lab tests (D-dimer, pro-thrombin, pro-thrombin time, comprehensive metabolic panel with
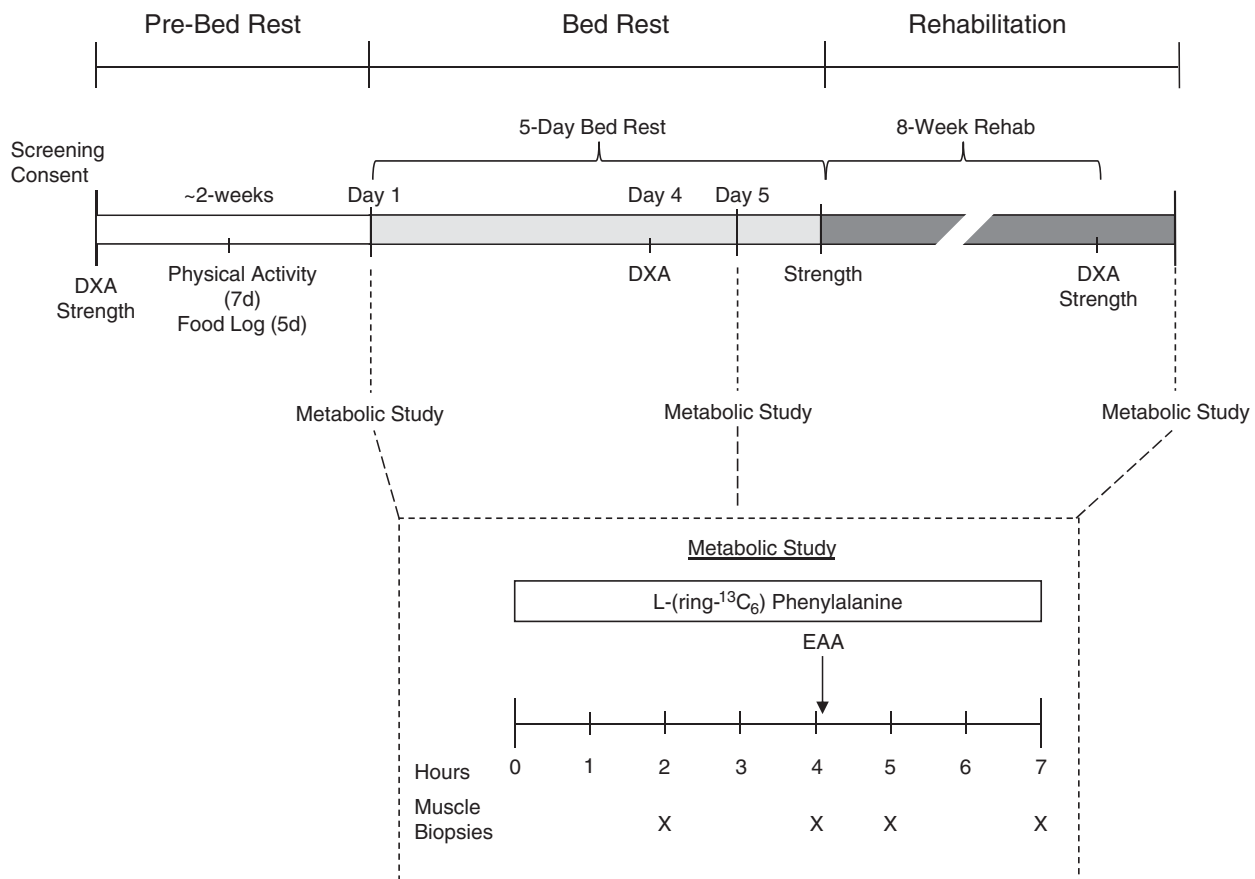

Figure 1. Study timeline and metabolic study
See Methods for details. Note: of the 14 young subjects that completed BED REST, only half (*n* = 7) of these subjects were randomized to complete the REHAB intervention.

platelet differential) were taken in the early morning before and during bed rest (days 1–5) for monitoring subject safety. Following bed rest, a second metabolic study was performed but muscle biopsies were performed on the opposite leg as on day 1. Finally, a mental exam and brief physical test of activities of daily living was performed to deem the subject safely discharged from the clinical research unit. Strength was repeated afterwards before beginning exercise rehabilitation while the DXA scan was repeated on bed rest day 4 for methodological purposes.

Exercise rehabilitation

We have reported the efficacy of high-intensity eccentric contractions on lower limb strength and hypertrophy in older adults (LaStayo *et al.* 2014). We intentionally chose an eccentric exercise rehabilitation regimen because of its reputation to deliver high force muscle contractions yet at a metabolically lower energy cost with application and feasibility in older adult rehabilitation populations (LaStayo *et al.* 2014). The 3× per week (every other day) exercise rehabilitation protocol was increased incrementally over 8 weeks to maintain intensity. Subjects were encouraged to maintain exercise intensity in the range of 13–16 (somewhat hard/hard) on a 6–20 point rating of perceived exertion Borg scale. For the first 2 weeks, subjects gradually increased exercise volume to 13 continuous minutes of eccentric exercise. The subsequent weeks (weeks 3–5), the workload was divided into three high intensity 5-min intervals with 1 min rest/interval. The final 3 weeks of training (weeks 6–8), volume was increase to four 5-min intervals (weeks 6–8). Total work time peaked at 20 min. Total work (kJ) was recorded after each exercise session to monitor progress. Following each exercise session, 17 g BCAA-enriched whey protein (4.6 g leucine, 2.4 isoleucine, 2.3 g valine; BCAA Pepform BCAA Peptide, Glanbia Nutritionals, Twin Falls, ID, USA) was consumed by the subject in the presence of an investigator. The post-exercise protein supplementation was strategically used to maximize the protein anabolic response to exercise as has been documented previously (Cermak *et al.* 2012). Training days were supervised and recorded by a trained exercise specialist (research coordinator) to monitor compliance and progress. All subjects were asked to maintain their day-to-day habitual activities (e.g. walking) and normal daily living and dietary habits during the exercise rehabilitation period. Adherence was indicated by missing no more than 2 of the 24 training sessions (~92%). After exercise rehabilitation, subjects repeated the DXA scan and strength test. Finally, subjects completed the last metabolic study, approximately a week following the last rehabilitation training session.

Measurement of mixed muscle protein synthesis

Postabsorptive and postprandial muscle tissue samples were ground, and intracellular free amino acids and muscle proteins were extracted as previously described (Dreyer *et al.* 2006) and were carried out at the University of Texas Medical Branch. Intracellular free concentrations L-[ring-$^{13}C_6$] phenylalanine enrichments were determined by gas chromatography–mass spectrometry (GCMS, 6890 Plus CG, 5973N MSD, 7683 autosampler, Agilent Technologies, Palo Alto, CA, USA) (Wolfe & Chinkes, 2005). Mixed-muscle protein-bound phenylalanine enrichment was analysed by GCMS after protein hydrolysis and amino acid extraction (Dreyer *et al.* 2006), using the external standard curve approach (Calder *et al.* 1992). Muscle protein synthesis was calculated by measuring the incorporation rate of the phenylalanine tracer into the proteins, and using the precursor-product model to calculate the synthesis rate as reported elsewhere (Drummond *et al.* 2012). Data were expressed as percentage per hour (% h^{-1}) and were calculated in fasted muscle tissue and over an EAA ingestion period (0–3 h).

Western blotting

The relative abundance of target proteins was determined in muscle samples via Western blotting as we have done previously (Drummond *et al.* 2013). Briefly, tissue samples were homogenized 1:10 (w/v) using a glass tube and mechanically driven pestle grinder in an ice-cold buffer containing 50 mM Tris (pH 7.5), 250 mM mannitol, 40 mM NaF, 5 mM pyrophosphate, 1 mM EDTA, 1 mM EGTA and 1% Triton X-100 with a protease inhibitor cocktail. Homogenates were centrifuged for 10 min at 4°C. After centrifugation, the supernatant was collected and the protein concentration determined using a modified Bradford protein assay and measured by a spectrophotometer (EPOCH; BioTek, Winooski, VT, USA). Proteins from the supernatant fraction were separated via polyacrylamide gel electrophoresis (7.5% or 12%), transferred onto a polyvinylidene difluoride membrane, and incubated with primary and secondary antibodies directed against the proteins of interest. Chemiluminescence reagent (ECL Plus, Thermo Scientific) was applied to each blot for 5 min. Membranes were exposed on a ChemiDoc XRS (Bio-Rad) and quantified with Image lab software (Bio-Rad). Membranes containing phospho-detected proteins were stripped (25 mM glycine, pH 2.0 and 1% SDS) of primary and secondary antibodies then re-probed for total protein. We also included the same loading control on each gel in order to correct for gel-to-gel variability. Data were normalized to internal control and reported as fold

change from baseline levels (basal or PRE), and therefore baseline was set to 1.

Antibodies

The specific antibodies (Cell signalling Technologies, Danvers, MA, USA) used to detect target proteins were: phospho-mTOR (Ser2448), total mTOR, phospho-S6K1 (Thr389), total S6K1, phospho-4EBP1 (Thr37/46), total 4EBP1, phospho-eEF2 (Thr56), total eEF2, phospho-rpS6 (Ser240/244), total rpS6, phospho-AMPKα (Thr172), total AMPKα, LC3II/I (reported as the ratio of LC3II/LC3I), and Beclin-1. Secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). GAPDH (Cell signalling Technologies) was used to verify equal protein loading in each lane.

RNA isolation, cDNA synthesis and real-time PCR

Step-by-step methods for each of these procedures can be found in a previous publication (Drummond *et al.* 2010). Total RNA was isolated by homogenizing 10–15 mg tissue with a hand-held homogenizer in a solution containing Tri reagent LS (Molecular Research Centre, Cincinnati, OH, USA). The RNA was separated and precipitated using chloroform and isopropanol. Extracted RNA was washed with ethanol then suspended in nuclease-free water with EDTA. RNA integrity was assessed in select samples using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The RNA integrity number was 8.8 ± 0.1 (1 to 10 scale; 10 = highest integrity). RNA concentration was determined using the EPOCH (Take3; BioTek) spectrophotometer. RNA was DNase-treated and cDNA was synthesized using commercially available kits (DNA-free, Ambion, Austin, TX, USA; iScript, BioRad, Hercules, CA, USA). All isolated RNA and cDNA samples were stored at −80°C until analysed.

Real-time qPCR was carried out with a CFX Connect real-time PCR cycler (BioRad) in combination with SYBR green fluorescence (iQ SYBR green supermix; BioRad). Cycle threshold values of target genes were normalized to beta 2-microglobulin (β2M) then fold change values were calculated ($2^{-\Delta\Delta Ct}$). Forward and reverse primer sequences for human MURF1, MAFBX, REDD1, REDD2, and β2M have been published previously (Drummond *et al.* 2008,2009). β2M remained stable across interventions and between age groups.

Statistical analysis

Change in lean mass and EAA-induced muscle protein synthesis from baseline to 5 days of bed rest were chosen as the primary endpoints with the main hypothesis that older subjects would be more sensitive to the effects of bed rest than younger subjects. Other measures (e.g. muscle mTORC1 signalling, proteolytic markers) were considered supportive or explanatory for this main hypothesis. Therefore, no adjustment was made to significance levels for other tests. Significance was thus pre-determined at $P < 0.05$. In general, statistical comparisons were made between the changes from baseline to after bed rest or after exercise rehabilitation and between young and older subjects at the same time point. The number of younger subjects randomized to the rehabilitation intervention was determined by hypothesizing that the young subjects would have no change (from pre-bed rest) in EAA-induced MPS after 5 days bed rest or rehabilitation while the older subjects would recover protein anabolic sensitivity with rehabilitation. Therefore, we determined that for a power of 80%, a sample size of 7 would allow us to detect differences of changes as a result of the rehabilitation intervention.

Descriptive statistics are presented as means $\pm$ SEM. Student's *t* tests were used to assess differences in baseline characteristics between young and older adults. Because our recruitment and randomization approach led to an unbalanced design, both with regard to the number in each group and for sex, we analysed all changes and intergroup comparisons with a repeated measures analysis of variance model using PROC MIXED (SAS Corporation, Cary, NC, USA). This model uses maximum likelihood methods to generate fixed effect estimates of least square means and standard errors for balanced or unbalanced data. Further, we tested for effects of sex, statins, and contraceptive use in separate models and found none of these covariates to be significant. In addition, due to the smaller group of young subjects that went on to the rehabilitation phase, we tested all comparisons with the rehabilitation phase of the study separately by restricting our subjects to those who participated in all phases. For simplicity of presentation, the rehabilitation phase is depicted with the baseline and bed rest phases in the same figures, though it was analysed separately. All analyses were performed with SAS version 9.4 (Cary, NC, USA).

Results

Subject characteristics

At PRE, 14 young (Y; 22 ± 1 years) and 9 older (O; 66 ± 1 years) subjects did not vary by body weight (Y; 67 ± 4 *vs.* O; 72 ± 4 kg), height (Y; 1.74 ± 0.02 *vs.* O; 1.71 ± 0.03 m), total lean mass (Y; 46 ± 3 *vs.* O; 43 ± 3 kg), bone mineral density (Y; 1.14 ± 0.02 *vs.* O; 1.05 ± 0.05 g cm^{-2}) or BMI (Y; 23 ± 1 *vs.* O; 25 ± 1 kg m^{-2}). However at PRE, total body fat was higher in older adults (Y; 18 ± 2 *vs.* O; 26 ± 2 kg) ($P < 0.05$). After bed rest, and only in older adults, body weight and total lean mass decreased by ~3% and ~4%, respectively ($P < 0.05$) but returned to PRE after REHAB. Total body

fat and bone density did not change after bed rest or after REHAB in young or older adults. At PRE, young and older subjects had similar daily physical activity levels as measured by the average step count over a 5-day period (Y; 5982 ± 113 vs. O; 5760 ± 73 steps) and had a similar 5-day average caloric intake (Y; 3247 ± 86 vs. O; 2945 ± 95 kcal).

Leg lean tissue and isometric strength (Fig. 2)

At PRE, young and older subjects had similar levels of leg lean mass (Y; 15.7 ± 0.9 vs. O; 13.4 ± 1.0 kg) and peak isometric leg extension strength (Y; 160 ± 6 vs. O; 134 ± 3 N m). However, after bed rest, older adults had reduced leg lean mass ($P < 0.0001$) and strength ($P = 0.02$) compared to before bed rest ($P < 0.05$).

Table 1. Pre-bed rest basal expression of proteolytic markers and mTORC1 inhibitors in young and older adult skeletal muscle

	Young	Old	P value
MURF1 mRNA	1.04 ± 0.09	1.86 ± 0.14	<0.01
MAFBX mRNA	1.11 ± 0.15	2.24 ± 0.32	<0.01
REDD1 mRNA	1.33 ± 0.29	2.68 ± 0.56	0.03
REDD2 mRNA	1.29 ± 0.29	1.58 ± 0.33	0.53
AMPKα (Thr172)	0.68 ± 0.23	0.90 ± 0.22	0.50
AMPKα protein	2.12 ± 0.90	1.90 ± 0.54	0.86
LC3II/I protein	0.75 ± 0.12	0.12 ± 0.04	<0.01
Beclin1 protein	8.06 ± 2.97	11.64 ± 3.83	0.46

Values are means ± SEM (young, $n = 14$; old, $n = 9$). Data for mRNA are reported as fold change calculated from $2^{-\Delta\Delta Ct}$. Protein expression data are reported in arbitrary units normalized to an internal control.

Figure 2. Changes in lean mass and strength with bed rest and rehabilitation
Data (means ± SEM) in figure represents the percentage change in leg lean mass and strength (compared to pre-bed rest levels) in young and older adults after bed rest and after exercise rehabilitation. Leg lean mass determined by dual-energy X-ray absorptiometry and strength was determined by peak isometric knee extension. *Significantly different from pre-bed rest ($P < 0.05$); #significantly different from Young ($P < 0.05$); §significantly different from BED REST ($P < 0.05$).

Reductions in leg lean mass in older adults after bed rest was significantly different from younger adults after bed rest ($P < 0.01$). After REHAB, in younger adults, leg lean mass increased above PRE ($P < 0.05$) whereas, for older adults, leg lean mass returned to PRE levels. Following REHAB, strength increased above bed rest in young and older adults ($P < 0.05$) to levels similar to PRE.

Exercise rehabilitation

All subjects had high compliance with the exercise rehabilitation intervention (Y 98%; O 99% retention). Young and older subjects increased exercise workload after each week during the first 4 weeks of training ($P < 0.05$). The workload plateaued and was maintained for the final 4 weeks of training in young adults whereas workload plateaued at week 6 for the older adults. The average exercise session workload (kJ) over the final 4 weeks (weeks 5–8) for the young adults was 60970 ± 6262 kJ. Average exercise session workload for young adults was significantly greater than older adults when compared to older adult exercise sessions averaged over the final four (38858 ± 3771 kJ) or three (40631 ± 3850 kJ) weeks ($P < 0.05$). However, when normalized to bed rest leg lean mass levels, the 4-week average exercise session workload between the age groups was similar (Y; 3.85 ± 0.47 vs. O; 3.04 ± 0.22 AU). The average rate of perceived exercise (RPE) was not different between young and older individuals over the course of 8 weeks of exercise rehabilitation (Y; 14.6 ± 0.2 vs. O; 14.1 ± 0.5; 6–20 scale). Normalized workload and RPE data indicate that older and younger adults exercised at similar exercise intensities.

Baseline mRNA and total protein expression (Table 1)

At PRE, baseline expression for skeletal muscle MURF1 (~190%), MAFBX (~225%) and REDD1 (~270%)

mRNA levels were significantly greater in older *versus* younger adults ($P < 0.05$). Additionally, the ratio of LC3II/I protein expression was ∼80% lower in older *versus* younger adults ($P < 0.05$). REDD2 mRNA and AMPKα (Thr172), total AMPKα and Beclin1 protein levels were not different between the age groups.

Skeletal muscle mTORC1 nutrient signalling and REDD1 and REDD2 gene expression (Fig. 3)

Skeletal muscle mTOR (Ser2448) and 4EBP1 (Thr37/46) increased at 1 h after EAA ingestion in both age groups ($P < 0.05$; Fig. 3*A* and *C*). These responses were similar across treatments. S6K1 and rpS6 phosphorylation increased in response to EAA in young and older adults at PRE and REHAB. However, S6K1 (Thr389) and rpS6 (Ser240/244) (Fig. 3*B* and *D*) EAA-induced phosphorylation at BED REST (1 hEAA) was significantly lower for both young and older adults ($P < 0.05$). Skeletal muscle S6K1 and rpS6 phosphorylation increased in young and older adults in some cases at 3 h EAA (*vs.*basal; $P < 0.05$). Skeletal muscle eEF2 (Thr56) phosphorylation did not change in response to EAA or across treatments or between the ages (data not shown). Total protein expression for skeletal muscle mTOR, 4EBP1, S6K1, rpS6 or eEF2 was not altered as a result of acute EAA, across treatments or between age groups (not shown). REDD1 mRNA expression increased in younger adults after BED REST and remained higher than older adults after REHAB (Fig. 3*E*) ($P < 0.05$). Finally, skeletal muscle REDD2 mRNA abundance increased by ∼200% in both young and older adults after BED REST (Fig. 3*F*) ($P < 0.05$) but returned to PRE levels after REHAB. mTOR (Ser2448) in a subset of young subjects for PRE have been published previously (Carlin *et al.* 2014).

Molecular regulators of muscle proteolysis and AMPKα phosphorylation (Fig. 4)

We found MURF1 mRNA was significantly increased in young and older adults after BED REST ($P < 0.05$) and remained elevated in young adult skeletal muscle after REHAB ($P < 0.05$). Following REHAB, MURF1 mRNA returned to PRE levels in older adults. On the other hand, MAFBX mRNA (Fig. 4*B*) and AMPKα (Thr172) (Fig. 4*C*) only increased in older adults after BED REST ($P < 0.05$) while AMPKα (Thr172) decreased in the young, MAFBX mRNA and AMPKα phosphorylation was different between the age groups after BED REST (MAFBX: $P = 0.04$; AMPKα: $P < 0.001$). Additionally, after BED REST, the ratio of LC3II/I (Fig. 4*D*) was increased only in older adults ($P < 0.0001$) and was different from the young ($P < 0.001$). Interestingly, the LC3II/I ratio further increased after REHAB in older adults ($P < 0.01$), reaching an absolute expression level that was

comparable to the young (absolute LC3II/I ratio levels: PRE: Y; 0.75 ± 0.12 *vs.* O; 0.12 ± 0.04 AU, BED REST: Y; 0.62 ± 0.10 *vs.* O; 0.24 ± 0.09 AU, REHAB: Y; 0.81 ± 0.20 *vs.* O; 0.49 ± 0.12 AU). Finally, there were no changes in Beclin1 protein expression levels in young or older adults or as a result of treatment (Fig. 4*E*). Representative Western blot images for mTORC1 nutrient signalling proteins and AMPKα, LC3II/I ratio, and Beclin1 protein expression can be found in Fig. 5.

Mixed-muscle protein synthesis (Fig. 6)

There were no differences in postprandial mixed-muscle protein synthesis rates between the age groups or across treatments. Young and older adults increased muscle protein synthesis after EAA ingestion (0–3 h) at PRE and after REHAB ($P < 0.05$). Although, EAA increased muscle protein synthesis in younger muscle after BED REST ($P < 0.05$) this did not occur in older adult skeletal muscle (BED REST: Y 0–3 hEAA *vs.* O 0–3 hEAA; $P = 0.01$).

Discussion

This is the first study to show that the loss in lean tissue and strength during the early stages of bed rest is age dependent. Unlike healthy younger adults who largely were resistant to the short-term disuse stimulus, healthy older adults, with similar BMI and prior physical activity, lost nearly 4% lower limb muscle mass and ∼16% knee extensor isometric strength. Moreover, amino acid-induced protein synthesis rates were blunted and markers of muscle proteolysis (AMPKα, MAFBX and LC3II/I ratio) were higher in older adults after bed rest. Secondly, we observed in older individuals that rehabilitation following bed rest, as defined by a structured eccentric resistance training programme (combined with BCAA-enriched protein supplementation), reversed muscle functional deficits and restored muscle nutrient anabolic sensitivity to pre-bed rest levels. Together, these novel data indicate that older adults are more prone to muscle mass deterioration during the initial stages of bed rest, a mechanism that may be associated with a dysregulation of nutrient-induced protein anabolism and perhaps to a lesser extent increased levels of molecular regulators related to skeletal muscle protein breakdown. Finally, bed rest-induced muscle impairments in healthy older adults are restored with an intensive 8-week exercise rehabilitation period.

To provide a mechanism for age-related changes in lean tissue with bed rest, we measured skeletal muscle mTORC1 nutrient signalling and mixed-muscle protein synthesis in response to acute EAA ingestion in young and older subjects. Consistent with our previous work after 7 days of bed rest (Drummond *et al.* 2012), mTORC1
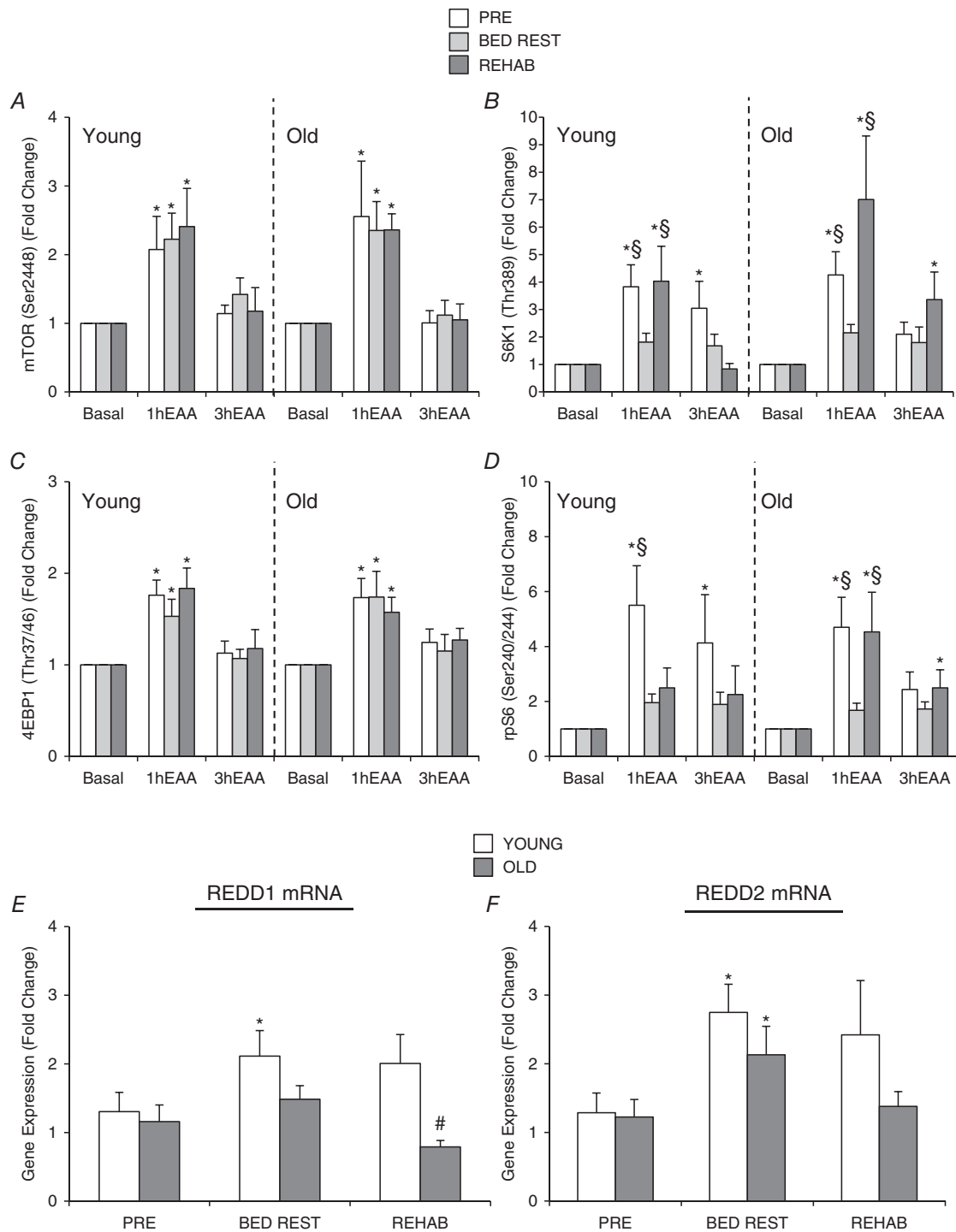
Figure 3. Muscle mTORC1 Nutrient Signalling and REDD gene expression

Data (means ± SEM) represents protein expression for: mTOR (Ser2448; *A*), S6K1 (Thr389; *B*), 4EBP1 (Thr37/46; *C*), and rpS6 (Ser240/244; *D*) at basal and 1 and 3 h after acute EAA ingestion repeated before bed rest (PRE, white bar), after 5 days bed rest (BED REST, light grey bar) and after 8 weeks exercise rehabilitation (REHAB, dark grey bar). Panels *E* and *F* are basal mRNA expression for REDD1 and REDD2, respectively, at PRE, BED REST and REHAB study time points. Western blot data are reported as fold change from fasting levels (basal), and therefore were set to 1. Data for mRNA are reported as fold change from basal calculated from $2^{-\Delta\Delta Ct}$.*Significantly different from Basal at corresponding time point or PRE ($P < 0.05$); §significantly different from BED REST at corresponding time point ($P < 0.05$); #significantly different from Young.

nutrient signalling (e.g. S6K1 and rpS6 phosphorylation) and EAA-induced mixed-muscle protein synthesis was suppressed after 5 days bed rest in older adults. These changes were uniquely complemented with increased mRNA expression of the mTORC1 inhibitor REDD2 in both young and old after bed rest. A similar finding has been observed in rodents after immobilization (Kelleher *et al.* 2015), suggesting that REDD2 may be intricately tied to regulation of mTORC1 signalling with disuse in human skeletal muscle. Interestingly, REDD1 and REDD2 mRNA responses were higher and EAA-stimulated mTORC1 signalling was reduced in younger subjects but muscle protein synthesis (and lean mass) was unchanged with 5 days bed rest. We speculate that the discrepancy between
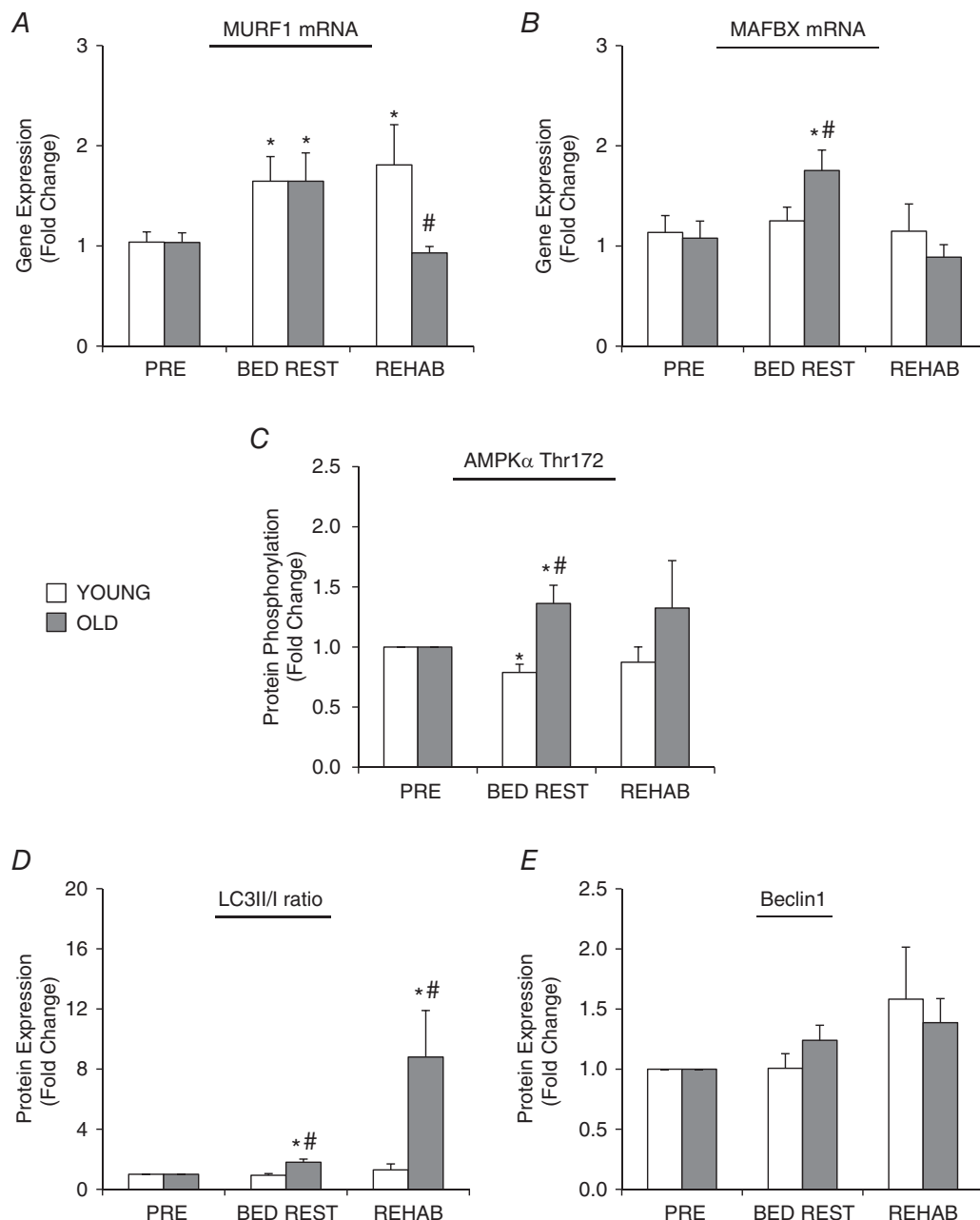
Figure 4. Muscle proteolytic markers and AMPKα

Data (means ± SEM) in Young (white bar) and Older (grey bar) adults represents: MURF1(*A*) and MAFBX(*B*) mRNA expression and protein expression for AMPKα (Thr172; *C*), LC3II/I ratio(*D*) and Beclin1(*E*) sampled in the fasted state before bed rest (PRE), after 5 days bed rest (BED REST) and after 8 weeks exercise rehabilitation (REHAB). Western blot data are reported as fold change from fasting levels and therefore were set to 1. Data for mRNA are reported as fold change from basal calculated from$2^{-\Delta\Delta Ct}$. *Significantly different from PRE ($P < 0.05$);#significantly different from Young at corresponding time point ($P < 0.05$).
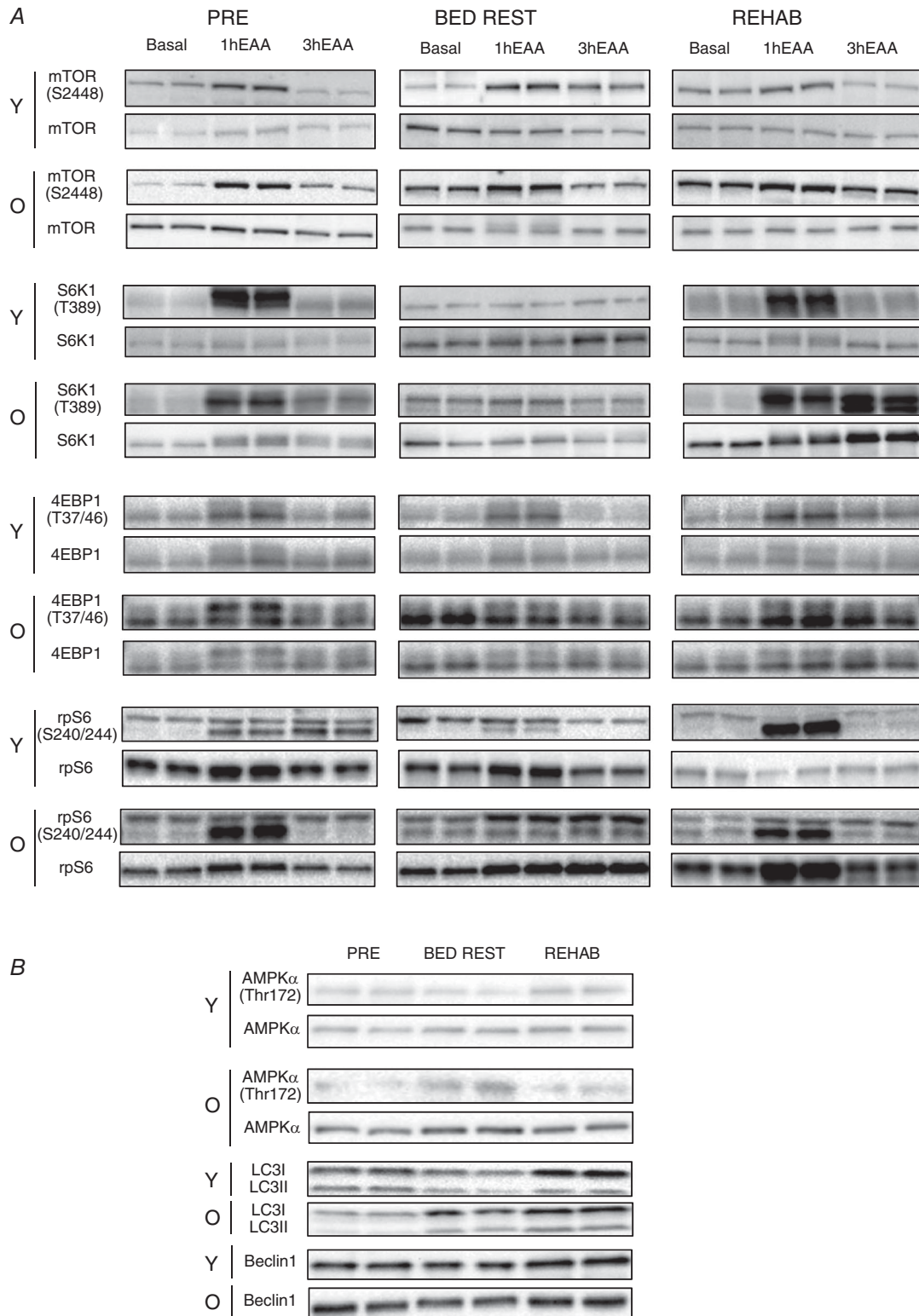
Figure 5. Representative Western Blot Images

A, Western blot images are EAA-induced mTORC1 nutrient signalling markers in Young (Y) and Older (O) adults skeletal muscle before bed rest (PRE), after 5 days bed rest (BED REST) and after 8 weeks of exercise rehabilitation (REHAB). Images (in replicate) are representative of fasted (basal) and 1 and 3 h EAA skeletal muscle responses following each intervention. *B*, representative western blot images (in replicate) for AMPKα and select proteolytic markers in Young (Y) and Older (O) adult skeletal muscle in the fasted state before (PRE), after bed rest (BED REST) and after exercise rehabilitation (REHAB).

molecular signalling events, protein synthesis and whole muscle changes in young individuals with 5 days of bed rest may be a result of time course, since prior studies have noted that at least 7 days of bed rest is required for noticeable changes in muscle mass to be observed in the young (Ringholm *et al.* 2011).

Our findings suggest that changes in muscle size and strength in older adults during short-term bed rest may also be related to an age-dependent increase in markers related to muscle protein breakdown. This hypothesis has been suggested in a recent review article (Wall *et al.* 2013*a*). In our study, older adult skeletal muscle after bed rest was uniquely characterized by a modest increase in skeletal muscle MAFBX mRNA abundance and LC3II/I protein ratio, an expression pattern that was not observed in younger adult skeletal muscle. The ubiquitin proteasome system and autophagy are well accepted as major muscle proteolytic pathways that regulate muscle cell size (Sandri, 2013; Bodine & Baehr, 2014) and are activated particularly during the first few days of muscle disuse in humans (Reich *et al.* 2010; Suetta *et al.* 2012; Wall *et al.* 2013*a*). For example, Wall *et al.*(2014)demonstrated that 5 days of cast immobilization in young adults increased skeletal muscle MURF1 and MAFBX mRNA but only MURF1 returned to baseline levels after 14 days of immobilization in these same subjects. Similarly, we show an increase in skeletal muscle MURF1 mRNA in young and older adults after 5 days of bed rest but a nearly 200% increase in MAFBX mRNA only in older adults, suggesting that additional myofibrillar and sarcolemmal proteins may be targeted for degradation during bed rest in the older adult.

Few studies have examined the autophagy–lysosomal system with bed rest. Brocca and colleagues showed that 24 days of bed rest in young healthy adults increased muscle Beclin1 mRNA (Brocca *et al.* 2012). Although we did not identify skeletal muscle changes in Beclin1 protein levels, perhaps as a result of early (current study) *vs.* late (Brocca *et al.*) bed rest measurements, the ratio of LC3II/I protein expression increased, implying that there may be an increased rate of removal of organelles and protein aggregates in older adults during bed rest. In is unclear what may be mediating increased autophagy selectively in older adults but an increased AMPKα phosphorylation with bed rest noted only in older adult skeletal muscle might have played a role (Greer *et al.* 2007; Kim *et al.* 2011). On closer inspection of the LC3II/I data when re-plotted as absolute levels, older adults had reduced autophagy levels (e.g. decreased LC3II/I ratio) in comparison to the young before bed rest; an ageing phenomenon that has been observed by others (Wohlgemuth *et al.* 2010; Drummond *et al.* 2014). However, following an intensive resistance exercise programme in the old, muscle autophagy (LC3II/I ratio) was restored to youthful levels, suggesting that a dysfunctional autophagy–lysosomal system in ageing muscle cells may be reversible.

Another major finding of our study was that rehabilitation after a 5-day bed rest period successfully restored skeletal muscle function (muscle and strength) and muscle protein anabolism (protein synthesis, mTORC1 signalling) in older adults to pre-bed rest levels. It should be little surprise that 8 weeks of a high intensity resistance exercise programme did not increase lean mass or strength beyond pre-bed rest levels in older adults considering that older adults began the rehabilitation training programme with a significant leg muscle and strength deficit. However, when comparing muscle functional changes following bed rest and the rehabilitation programme, muscle and strength gains in young and older adults were quite comparable. These data suggest that healthy older adults retain the
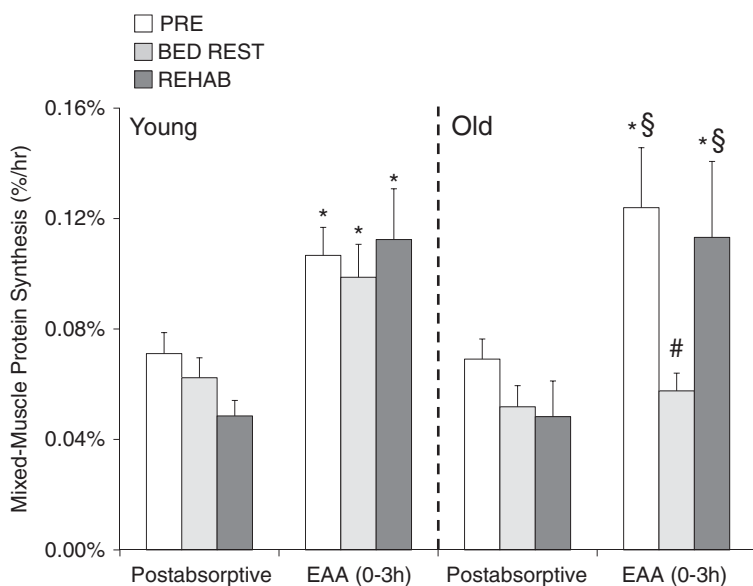

Figure 6. Muscle protein synthesis
Mixed muscle protein synthesis (means ± SEM) in skeletal muscle of young and older adults in the postabsorptive state (basal) and following essential amino acid (EAA) ingestion (0–3 h post EAA) at PRE (white bar), after BED REST (light grey bar) and after REHAB (dark grey bar).*Significantly different from respective postabsorptive value ($P < 0.05$);§significantly different from BED REST in response to EAA ($P < 0.05$);#significantly different from Young at BED REST.

capacity to regain muscle and strength like their younger counterparts after an inactivity period such as short-term bed rest. However it is possible that, in the early stages of rehabilitation after bed rest (weeks 1–4), older adults may have a delayed impaired regenerative response as has been demonstrated during recovery from limb immobilization (Suetta *et al.* 2013), but this idea remains to be tested in older adults following a period bed rest.

Finally, many bed rest studies of >7 days document that young individuals lose muscle mass (LeBlanc *et al.* 1992; Ferrando *et al.* 1996; Trappe *et al.* 2008; Ringholm *et al.* 2011; Rezen *et al.* 2014). Thus, it was surprising that young adults in this study did not lose leg lean mass after 5 days of bed rest, especially since MURF1 mRNA was elevated. This is in contrast to a recent short-term disuse study in young adults that reported reduced quadriceps volume after 5 days of cast immobilization (Wall *et al.* 2014). Moreover, Suetta and workers showed that young and older adults had a comparable decrease in fibre size at 4 days of limb immobilization, but at 14 days younger individuals had a greater relative muscle loss (Suetta *et al.* 2009,2012). These discordant findings may be a result of attempting to compare two unique disuse models. For example, bed rest delivers a whole body-deconditioning stimulus while cast/knee brace immobilization is localized to the involved limb. Secondly, subjects on bed rest were freely able to bend their limbs and also received daily passive range of motion of all lower limb joints by a physical therapist while cast immobilization involves complete joint immobilization. Perhaps the mild passive muscle activity (passive range of motion, intermittent compression devices) that was designed as standard of care during bed rest to mimic a hospital setting was sufficient to maintain protein anabolic sensitivity and slow muscle atrophy in young adults. Indeed, younger (*versus* older) adults have greater protein anabolic sensitivity to a bout of resistance exercise (Fry *et al.* 2011). It has also been suggested that the younger male subjects in prior immobilization studies experienced earlier/similar reductions in lean tissue with disuse compared to older adults because they started with a larger volume of muscle mass than the old (Wall *et al.* 2015). In contrast, our study subjects were made up of male and female young and older adults with comparable leg lean masses before bed rest.

There are some limitations to the current work including possibly not having sufficient power to detect changes in postabsorptive MPS. Additionally, some subjects were on statins and contraceptives, which may have altered muscle molecular events and protein metabolism (Hanai *et al.* 2007; Hansen *et al.* 2011). However, further statistical analysis showed that the effects of statins and contraceptives were not significant. Secondly, proteolytic activity assays should be assessed to further validate the role of muscle protein breakdown in contributing to aged-related muscle loss following short-term bed rest.

Finally, we acknowledge a limitation of this study was not examining fibre-specific cross sectional area (Suetta *et al.* 2013) since small changes in fibre area in the young subjects or important age-related changes after bed rest may have been masked by using a DXA scan.

In summary, 5 days of bed rest decreased leg lean tissue mass and knee extensor strength in older adults. Age-related differences in muscle mass may be attributed to a combined decrease in nutrient-induced protein synthesis and a moderate increase in markers of muscle proteolysis in the early stages of bed rest. The rapid loss in muscle and strength with 5 days of bed rest underscores the clinical concern for many older adults who are hospitalized, especially those who have limited muscle reserve prior to finding themselves on bed rest. Finally, older adult skeletal muscle fully recovers from bed rest-induced muscle deficits following a high-intensity eccentric exercise programme.

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Additional information

Competing interests

No authors declare a conflict of interest.

Author contributions

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