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RESEARCH ARTICLE

Effects of β-hydroxy-β-methylbutyrate on skeletal muscle mitochondrial content and dynamics, and lipids after 10 days of bed rest in older adults

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Standley RA, Distefano G, Pereira SL, Tian M, Kelly OJ, Coen PM, Deutz NE, Wolfe RR, Goodpaster BH. Effects of β-hydroxy-β-methylbutyrate on skeletal muscle mitochondrial content and dynamics, and lipids after 10 days of bed rest in older adults. *J Appl Physiol* 123: 1092–1100, 2017. First published July 13, 2017; doi: 10.1152/japplphysiol.00192.2017.—Loss of muscle mass during periods of disuse likely has negative health consequences for older adults. We have previously shown that β-hydroxy-β-methylbutyrate (HMB) supplementation during 10 days of strict bed rest (BR) attenuates the loss of lean mass in older adults. To elucidate potential molecular mechanisms of HMB effects on muscle during BR and resistance training rehabilitation (RT), we examined mediators of skeletal muscle mitochondrial dynamics, autophagy and atrophy, and intramyocellular lipids. Nineteen older adults (60–76 yr) completed 10 days BR followed by 8-wk RT rehabilitation. Subjects were randomized to either HMB (3 g/day HMB; $n = 11$) or control (CON; $n = 8$) groups. Skeletal muscle cross-sectional area (CSA) was determined by histology from percutaneous vastus lateralis biopsies. We measured protein markers of mitochondrial content [oxidative phosphorylation (OXPHOS)], fusion and fission (MFN2, OPA1, FIS1, and DRP1), autophagy (Beclin1, LC3B, and BNIP3), and atrophy [poly-ubiquinated proteins (poly-ub)] by Western blot. Fatty acid composition of several lipid classes in skeletal muscle was measured by infusion-MS analysis. Poly-ub proteins and OXPHOS complex I increased in both groups following BR ($P < 0.05$, main effect for time), and muscle triglyceride content tended to increase following BR in the HMB group ($P = 0.055$). RT rehabilitation increased OXPHOS complex II protein ($P < 0.05$), and total OXPHOS content tended ($P = 0.0504$) to be higher in HMB group. In addition, higher levels of DRP1 and MFN2 were maintained in the HMB group after RT ($P < 0.05$). BNIP3 and poly-ub proteins were significantly reduced following rehabilitation in both groups ($P < 0.05$). Collectively, these data suggest that HMB influences mitochondrial dynamics and lipid metabolism during disuse atrophy and rehabilitation.

NEW & NOTEWORTHY Mitochondrial content and dynamics remained unchanged over 10 days of BR in older adults. HMB stimulated intramuscular lipid storage as triacylglycerol following 10 days of bed rest (BR) and maintained higher mitochondrial OXPHOS content and dynamics during the 8-wk resistance exercise rehabilitation program.

mitochondria; bed rest; aging; HMB; exercise

OLDER ADULTS LOSE more muscle mass during a shorter period of bed rest (BR) than younger individuals who undergo longer BR duration. During 10 days of BR older adults lose 1 kg (28) of leg lean mass, while younger individuals only lose 0.4 kg after 28 days of BR (38). These findings highlight the need for effective countermeasures for older adults who undergo short periods of disuse. Nutritional interventions during BR with branched chain amino acids and/or complete nutrition are attractive strategies to preserve skeletal muscle mass due to their potent anabolic effect in skeletal muscle (11, 12). Other compounds have also garnered attention due to their preclinical and clinical effects on protein metabolism. Specifically, β-hydroxy-β-methylbutyrate (HMB) has been found to significantly impact skeletal muscle protein metabolism by simultaneously stimulating protein synthesis and reducing protein breakdown (56). We have previously reported that consuming HMB during 10 days of BR preserved lean body mass in older adults and improved muscle strength compared with placebo control during an 8-wk resistance training rehabilitation program following BR (16). These findings coupled with other HMB studies (6, 20, 34, 53) show that supplementation with HMB alone or with amino acids has a beneficial effect on skeletal muscle mass, strength, and function in older adults (57).

HMB is a naturally occurring metabolite of leucine generated by a two-step process starting with the transamination of leucine to α-ketoisocaproate (KIC) and further conversion by of KIC to HMB via a KIC-dioxygenase (37). Animal (preclinical) studies provide evidence that HMB exerts its positive effects on skeletal muscle protein metabolism through the stimulation of muscle protein synthesis (MPS) by activating mechanistic target of rapamycin-eukaryotic translation initiation factor 4E-binding protein 1-ribosomal protein S6 kinase β-1 (mTOR-4EBP1-p70SK1) pathway (5, 19, 26) and suppresses muscle protein breakdown (MPB) by inhibiting the ubiquitin proteasome pathway (19, 25, 29, 48, 49), inflammatory cytokines, and autophagy (19, 22). In addition, HMB has been shown to enhance recovery from immobilization by reducing myonuclear apoptosis and stimulating satellite cell regeneration in aged rats (3). These findings in animals are supported by an isotope study in humans showing HMB

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supplementation increases MPS and inhibits MPB in young healthy men (56). In addition to the positive effects on protein metabolism, recent evidence suggests that HMB can stimulate mitochondrial biogenesis and fatty acid oxidation via stimulation of peroxisome proliferator-activated receptor-γ coactivator-1α (24) and increase citrate synthase activity (40). These new mechanisms of action for HMB may be significant as emerging evidence suggests that mitochondrial dysfunction and reactive oxygen species (ROS) production may contribute to skeletal muscle atrophy.

Mitochondrial dysfunction during physical inactivity or immobilization manifests in multiple ways including the release of proapoptotic factors (1, 33), morphological alterations (fission, swelling), and energy stress (43), leading to elevated ROS production. In addition, recent evidence suggests intramyocellular sphingolipids contribute to ROS production in skeletal muscle (21). Oxidative stress stimulates muscle atrophy by increasing the expression of proteins involved in the autophagy and proteasome system (4, 15, 30, 35), activating the calpain and caspase-3 pathways (15, 35, 47, 55), modifying proteins making them susceptible to proteolytic degradation (39, 46, 58), and depressing protein synthesis (39, 46, 58). To our knowledge there is only one investigation examining the effects of short-term BR (7 days) on mitochondrial content, oxidative capacity and stress, and skeletal muscle lipids (17). Dirks et al. (17) found a reduction in mitochondrial oxidative phosphorylation (OXPHOS) protein content, citrate synthase, and β-hydroxyacyl-CoA dehydrogenase (β-HAD) enzyme activity in young men (mean age 23 yr). However, there were no changes in 4-hydroxynonenal, protein carbonylation, superoxide dismutase 2, catalase, and various lipid classes and species, suggesting the mechanism of muscle loss is independent of oxidative stress (2). This study does provide evidence that BR-induced alterations to mitochondrial content and oxidative capacity may occur concomitantly with a loss of quadriceps cross-sectional area (CSA; 3.2%) in younger individuals and highlights a need for effective countermeasures to help preserve skeletal muscle health during periods of disuse.

Our previous paper showed that HMB supplementation was effective at preserving lean body mass after 10 days of BR in older adults and enhanced strength recovery during an 8-wk RT rehabilitation program (16). Some proposed mechanisms underlying the beneficial effects of HMB could be via its effect on mitochondria, autophagy, and lipid metabolism. Thus the purpose here was to examine changes to muscle mitochondrial dynamics and autophagy and intramyocellular lipids following 10 days of BR and 8 wk of resistance training rehabilitation in older adults.

METHODS

Study design. This study was a prospective, randomized, double-blinded, placebo-controlled 10-wk investigation in older adults (n = 19, 60–76 yr). During the 10 wk, subjects completed 10 days of BR followed by an 8-wk progressive resistance training rehabilitation (RT) program of the upper and lower extremities three times per week and consumed a placebo (CON; n = 8, 1 male/7 female; age: 67 ± 2 yr; and body mass index (BMI): 26.5 ± 1.2 kg/m^2] or calcium HMB (3.0 g/day HMB; n = 11, 3 male/8 female; age: 67 ± 1 yr; and BMI: 24.9 ± 1.0 kg/m^2) throughout the study period. The study was conducted at the University of Arkansas for Medical Sciences Clinical Research Center, Little Rock and approved by the Institutional Review Board of University of Arkansas for Medical Sciences. All study procedures, risks, and benefits were explained to the subjects before giving written consent to participate. There was no significant difference between groups at baseline for the following variables: age (yr), BMI (kg/m^2), 25-OH-vitamin D (ng/ml), body weight (kg), total body fat (kg), bone mineral density (g/cm^2), fasted glucose (mg/dl), total cholesterol (mg/ll), serum albumin (g/dl), C-reactive protein (CRP; mg/l), and Short Performance Physical Battery score. A detailed presentation of the subject characteristics, study design, inclusion and exclusion criteria, supplementation, compliance, BR, body composition, strength testing, and resistance-exercise training measurements and related findings have been reported previously (16).

Skeletal muscle biopsy. Subjects underwent a muscle biopsy of the medialis vastus lateralis before and on the last day of BR and 24 h following the last bout of exercise at the end of the RT program. A biopsy sample was taken 10–15 cm above the knee under local anesthesia (lidocaine HCl 1%) with a 5-mm Bergstrom needle with suction. After the biopsy, excess blood, visible fat, and connective tissue were removed from the muscle tissue, and immediately frozen in liquid nitrogen (−190°C) until analysis. Since there was variability in the amount of sample that was obtained from each subject, not all subjects biopsies could be analyzed for all measures.

Histology. Histochemical analyses were performed on serial sections using methods previously described (7). Briefly, muscle was placed vertically in mounting medium on cork. Once on the cork, the muscle was frozen in isopentane cooled with liquid nitrogen until thoroughly frozen. Samples were placed in labeled cryotubes and stored in liquid nitrogen or −80°C until sectioning. Biopsy samples were sectioned (10 μm) and air-dried overnight and fixed in 25% acetone/75% ethanol for 5 min before staining. Sections were sequentially reacted immunohistochemically with antibodies to type I (Sigma clone NOQ7.5.4D monoclonal anti-myosin skeletal slow) and type II (Sigma clone MY32 monoclonal anti-myosin skeletal fast-alkaline phosphatase conjugated) myosin. Vector SG and Vector Red were used as the chromogens to detect type I and type II fibers, respectively. Images from the transverse muscle sections were captured using a microscope-mounted digital camera (Olympus BX-41 microscope and DP-71 camera). Images were analyzed using the Visiomorph (Visiopharm, Medicon Valley, Denmark) image analysis system. An average number of 256 fibers were analyzed per subject.

Western blot. Muscle homogenates were prepared as previously described (35). Proteins were separated by gel electrophoresis using a 4–20% gel (Bio-Rad Mini-PROTEAN TGX Precast Gel) and transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA). Membranes were blocked in 5% nonfat milk, and incubated with the following primary antibodies overnight at 4°C: DRP1 and OPA1 (1:500; BD Biosciences, San Jose, CA), FIS1 (1:1,000; Enzo Life Sciences, Farmingdale, NY), MFN2 and BNIP3 (1:500, 1:1,000; Sigma-Aldrich, St. Louis, MO), Beclin1 and LC3A/B (1:1,000; Cell Signaling, Danvers, MA), OXPHOS (1:1,000; MitoSciences, Eugene, OR), and α-tubulin (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were then incubated in appropriate species-specific secondary antibodies for 1 h (IRDye 800CW anti-Rabbit IgG No. 926–32211 and IRDye 680RD anti-Mouse IgG No. 926–68070; Li-Cor Biosciences, Lincoln, NE). Protein bands were visualized using a Li-Cor Odyssey infrared imaging system (Li-Cor Biosciences) and analyzed with Image Studio v2.1 software (Li-Cor Biosciences). Protein loading was controlled by normalizing bands of interest to α-tubulin expression. Gel-to-gel variation was controlled for by using a standardized sample on each gel.

To analyze poly-ub proteins, 20 ug of muscle samples were loaded on a 4–20% gel (Bio-Rad, Mini-PROTEAN TGX Precast Gel) as previously described (9). Proteins were transferred onto a nitrocellulose membrane overnight at 30 mA at 4°C. Membranes were blocked with 5% nonfat milk for 1 h and then incubated with primary antibody overnight at 4°C (anti-polyubiquitin; 1:1,000; Enzo Life Sciences, Farmingdale, NY). Membranes were then incubated with secondary

Table 1. *Skeletal muscle cross-sectional area*

	Control			HMB		
Cross-Sectional Area, μm^2	Pre (n = 8)	Post (n = 8)	Recovery (n = 7)	Pre (n = 10)	Post (n = 10)	Recovery (n = 10)
Type I	4,841 ± 617	5,073 ± 463	4,054 ± 510	5,071 ± 392	5,075 ± 496	5,108 ± 823
Type II	2,679 ± 385	3,444 ± 418	3,121 ± 389	3,377 ± 273	3,477 ± 467	3,585 ± 243

Data are means ± SE. HMB, β-hydroxy-β-methylbutyrate. No significant changes were observed in either group.

antibody for 1 h (IRDye 800CW anti-mouse IgM No. 925–32280; Li-Cor Biosciences). Protein bands were visualized as described above. The amount of poly-ub proteins was normalized to tubulin.

Lipidomics. Lipids were extracted from muscle samples (50 mg) in methanol:dichloromethane in the presence of 15:1n5, 17:0, 17:1n7, and d31SM16:0 for sphingomyelin as internal standards. The extracts were concentrated under nitrogen and reconstituted in 0.25 ml of 10mM ammonium acetate dichloromethane:methanol (50:50). The extracts were transferred to inserts and placed in vials for infusion-MS analysis, performed on a Shimazdu LC with nano PEEK tubing and the Sciex SelexIon-5500 QTRAP. The samples were analyzed via both positive and negative mode electrospray. The 5500 QTRAP scan was performed in MRM mode with the total of more than 1,100 MRMs. Individual lipid species were quantified by taking the peak area ratios of target compounds and their assigned internal standards and then multiplying by the concentration of internal standard added to the sample. Lipid class concentrations were calculated from the sum of all molecular species within a class, and fatty acid compositions were determined by calculating the proportion of each class comprised by individual fatty acids.

Statistical analysis. Comparisons between pre- and post-BR and post-BR and recovery were analyzed using two-way (group and time) ANOVA with repeated measures, and post hoc comparisons were made with Tukey's test. ANOVA testing was conducted on the fold change for protein and triacylglycerol (TAG) data and on absolute values for the lipid data in the Supplemental Tables S1–S11 (Supplemental Material for this article is available online at the Journal website). Normality of the data was tested using Shapiro-Wilk test, and nonnormally distributed data were log transformed. Significance was accepted at $P < 0.05$. Data are presented as means ± SE and analyzed using JMP version 13.0 (SAS Institute, Cary, NC).

RESULTS

Histology. Skeletal muscle CSA are presented in Table 1 and representative images in Fig. 1. There were no significant changes between groups in skeletal muscle CSA following BR in both groups (Type 1 CSA Interaction: $P = 0.49$; Type 2 CSA Interaction: $P = 0.14$).

Mitochondrial content. Mitochondrial OXPHOS protein content following BR and RT and representative blots are presented in Fig. 2, *A* and *B*. There was a significant main effect for time for complex I to increase in both groups (Interaction: $P = 0.74$; Time Effect: $P = 0.039$), and all other complexes remained unchanged following BR ($P > 0.05$).

There was a tendency for complex I to be reduced in CON after RT (Interaction: $P = 0.071$; Group Effect: $P = 0.095$). Complex II was significantly upregulated in the HMB group compared with CON following RT (Interaction: $P = 0.032$, post hoc CON recovery vs. HMB recovery: $P = 0.012$). There was a tendency for a total OXPHOS to be elevated in the HMB group after RT (Interaction: $P = 0.071$; Group Effect: $P = 0.0504$).

Mitochondrial dynamics and muscle proteolysis. The proteins associated with mitochondrial fission (FIS1 and DRP1), fusion (MFN2 and OPA1), and autophagy (Beclin1, LC3B,

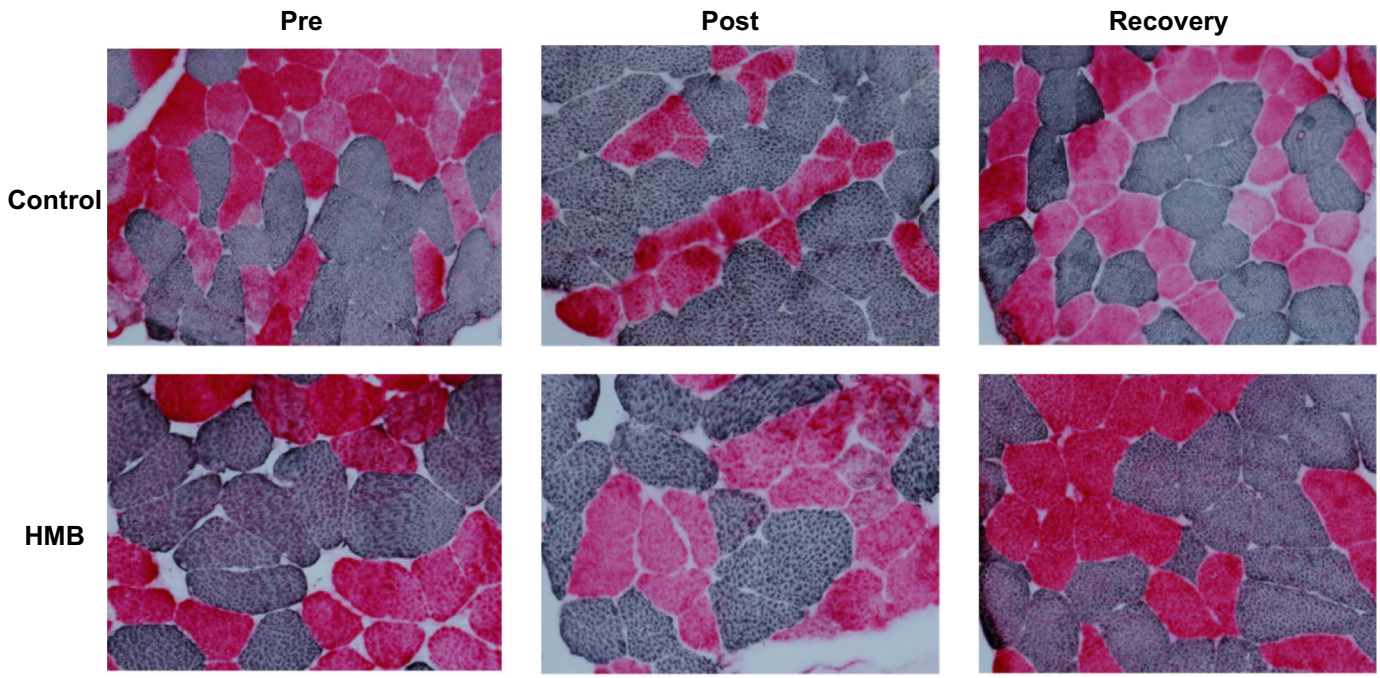

Fig. 1. Representative histology images for both control and β-hydroxy-β-methylbutyrate (HMB) groups at pre-bed rest (BR), post-BR, and recovery time points.

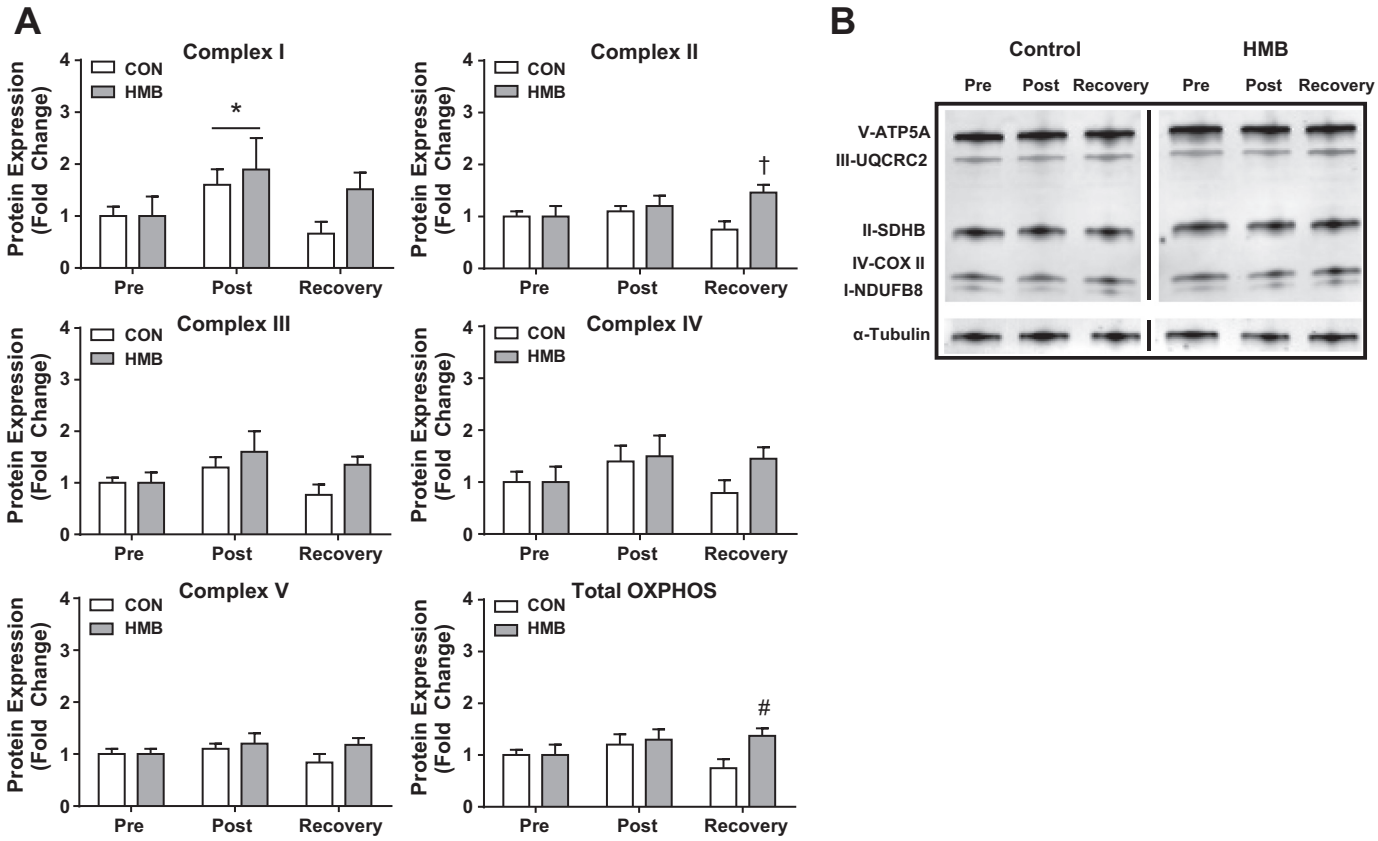

Fig. 2. Skeletal muscle mitochondria oxidative phosphorylation (OXPHOS) content pre- and post-10 days of bed rest (BR) and after an 8-wk resistance training rehabilitation (RT; *A*) and representative Western blot images (*B*). Pre- and post-BR data are reported as fold change from pre-BR and recovery data as fold change from post-BR. Vertical dividing lines were used in the Western blot images to present lanes from the same gel that were reorganized for presentation purpose; $n = 6–8$ per group; horizontal bars represent main effects, and error bars are SE. *Significant main effect for time from pre-BR (Interaction: $P = 0.74$; Time Effect: $P = 0.039$); #trend for group effect from pre-BR (Interaction: $P = 0.071$; Group Effect $P = 0.095$); †significantly different from control (CON) following RT (Interaction: $P = 0.032$, post hoc CON recovery vs. HMB recovery: $P = 0.012$); #trend for group effect following RT (Interaction: $P = 0.071$; Group Effect $P = 0.0504$).

and BNIP3) following BR and RT rehabilitation are presented in Fig. 3, *A* and *B*. There were no significant changes in mitochondrial fission (FIS1 and DRP1), fusion (MFN2 and OPA1), and autophagy (Beclin1, LC3B, and BNIP3) protein levels following BR. There was a significant increase in poly-ub proteins in both groups following BR (Interaction: $P = 0.81$; Time Effect: $P = 0.027$).

DRP1 was significantly elevated in the HMB group compared with CON (Interaction: 0.078; Group Effect: $P = 0.001$), and there was a significant group effect for MFN2 following RT (Interaction: $P = 0.73$; Group Effect: $P = 0.016$). There was a significant time effect for BNIP3 to be elevated (Interaction: $P = 0.33$; Time Effect: $P = 0.04$) and poly-ub proteins to be reduced following RT (Interaction: $P = 0.62$; Time Effect: $P = 0.0005$).

Lipidomics. Muscle total TAG (Interaction: $P = 0.123$; Time Effect: $P = 0.057$), and saturated (Interaction: $P = 0.124$; Time Effect: $P = 0.058$), monounsaturated (Interaction: $P = 0.118$; Time Effect: $P = 0.057$), and polyunsaturated (Interaction: $P = 0.136$; Time Effect: $P = 0.051$) TAG levels tended to increase in the HMB group following BR (Fig. 4, *A* and *B*). There were significant increases in omega-3 (n3)-length TAGs (Interaction: $P = 0.117$; Time Effect: $P = 0.044$) and trends for increases in n6 (Interaction: $P = 0.143$; Time Effect: $P = 0.053$) and n9 (Interaction: $P = 0.114$; Time Effect: $P = 0.058$) TAGs following BR in the HMB group (Fig. 4*C*). Several TAG species had trends to be increased following BR: C15:0 (Interaction: $P = 0.12$; Time Effect: $P = 0.11$), C18:0 (Interaction: $P = 0.319$; Time Effect: $P = 0.0504$), C18:3n-3 (Interaction: $P = 0.098$; Time Effect: $P = 0.051$), C18:2n-6 (Interaction: $P = 0.138$; Time Effect: $P = 0.0525$), C20:2n-6 (Interaction: $P = 0.152$; Time Effect: $P = 0.0515$), C18:1n-9 (Interaction: $P = 0.114$; Time Effect: $P = 0.057$), and C20:1n-9 (Interaction: $P = 0.095$; Time Effect: $P = 0.074$) (Fig. 3*D*). Several TAG species had significant increases following BR: C16:0 (Interaction: $P = 0.131$; Time Effect: $P = 0.043$), C22:5n-3 (Interaction: $P = 0.124$; Time Effect: $P = 0.022$), C22:6n-3 (Interaction: $P = 0.67$; Time Effect: $P = 0.020$), C22:5n-6 (Interaction: $P = 0.124$; Time Effect: $P = 0.019$), and C22:1n-9 (Interaction: $P = 0.487$; Time Effect: $P = 0.01$) (Fig. 4*D*). Total phosphatidylethanolamine tended to be reduced in the HMBS group following BR (Interaction: $P = 0.69$; Group Effect: $P = 0.08$; Supplemental Table S7). Total cholesteryl ester (Interaction: $P = 0.82$), diacylglycerols (Interaction: $P = 0.59$), fatty acids (Interaction: $P = 0.83$), cardiolipin (Interaction: $P = 0.90$), lysophosphatidylcholine (Interaction: $P = 0.33$), phosphatidylcholine (Interaction: $P = 0.22$), phosphatidylserine (Interaction: $P = 0.97$), sphingomyelin (Interaction: $P = 0.52$), and the phosphatidylcholine-to-phosphatidylethanolamine ratio (Inter-

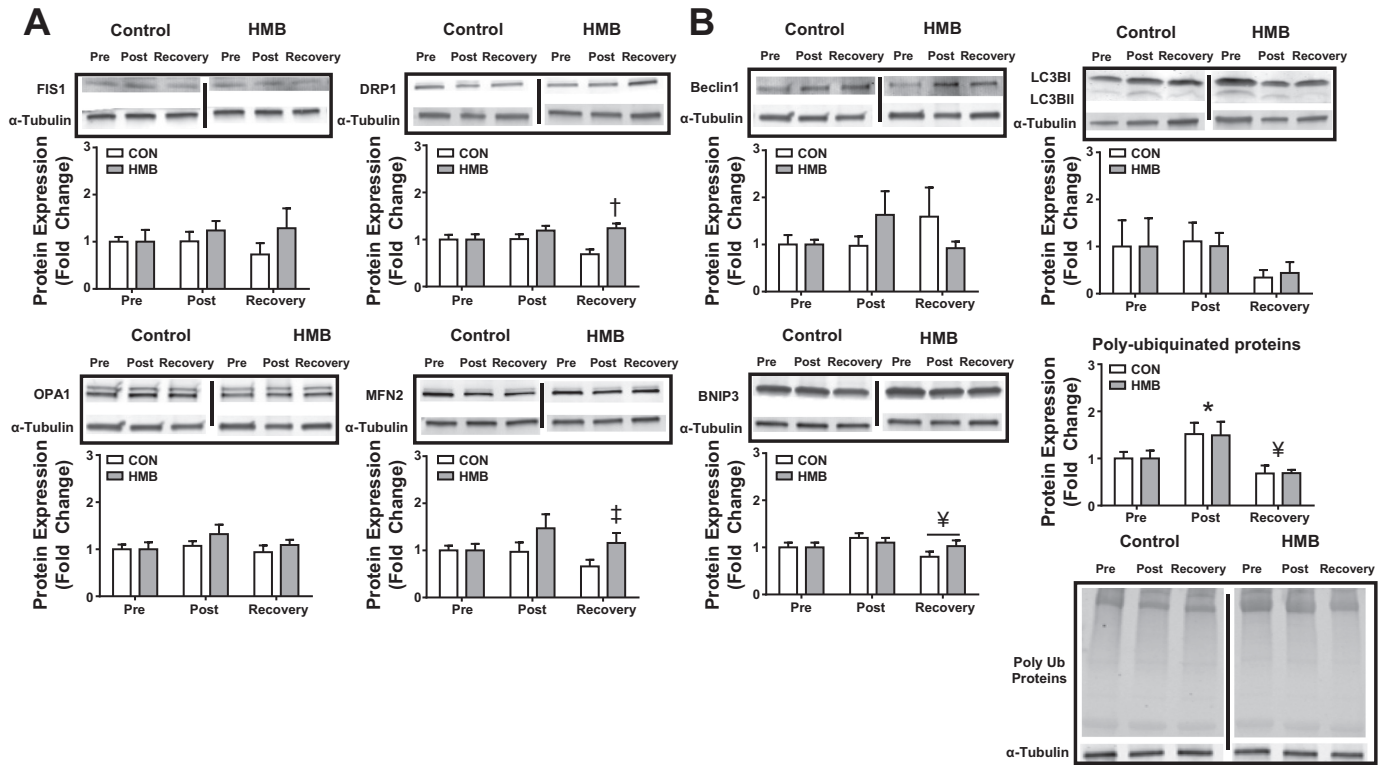

Fig. 3. Skeletal muscle mitochondria fission (FIS1 and DRP1) and fusion (OPA1 and MFN2; *A*) and autophagy (Beclin1, BNIP3, and LC3BII-to-LC3BI ratio) and atrophy [poly-ubiquinated (poly-ub)] proteins (*B*) pre- and post-10 days of BR and RT. Pre- and post-BR data are reported as fold change from pre-BR and recovery data as fold change from post-BR. Vertical dividing lines were used in the Western blot images to present lanes from the same gel that were reorganized for presentation purpose; $n = 6–8$ per group; horizontal bars represent main effects, and bars are SE. *Significant main effect for time from pre-BR (Interaction: 0.81; Time Effect: $P = 0.027$); †significantly different from CON following RT (Interaction: 0.078; Group Effect: $P = 0.001$); ‡significant main effect for group from RT (Interaction: $P = 0.73$; Group Effect: $P = 0.016$); ¥significant main effect for time from post-BR (BNIP3, Interaction: $P = 0.33$; Time Effect: $P = 0.04$; poly-ub, Interaction: $P = 0.62$; Time Effect: $P = 0.0005$).

action: $P = 0.11$) remained unchanged following BR and uninfluenced by HMB (Supplemental Tables S1–S11). Although the total contents of some lipid classes did not change, some individual lipid species were altered following BR (Supplemental Tables S1–s11).

DISCUSSION

Our previous study has shown that there is extensive loss of muscle mass over 10-day BR in older adults and that HMB could prevent or attenuate disuse atrophy during BR. To begin to understand the molecular mechanisms leading to muscle mass loss over BR and pathways impacted by HMB, we examined changes to muscle mitochondrial dynamics autophagy and the ubiquitin proteasome pathway following 10 days of BR and 8 wk of RT rehabilitation in older adults. In addition, we evaluated the effects of disuse atrophy and rehabilitation on changes in intramyocellular lipids and whether or not they were influenced by HMB. The main findings from this investigation are as follows: first, levels of proteins related to mitochondrial OXPHOS and dynamics remained unchanged in older adults following 10 days of BR in both groups despite differences in muscle mass loss between groups. Second, immobilization caused an increase in TAG in the muscle and this metabolic change was enhanced in the HMB-treated muscles that were preserved during BR. Third, during RT rehabilitation, HMB maintained higher mitochondrial OXPHOS content and proteins associated with mitochondrial fission (DRP1) and fusion (MFN2), which was not observed in the placebo group receiving RT rehabilitation.

BR period. We found no changes in skeletal muscle fiber CSA in both groups following 10 days of BR (Table 1). Interestingly, these changes occurred despite a significant reduction in leg lean mass in the CON group (16). These are similar findings to previous studies that found no changes in muscle fiber CSA with reductions in leg lean mass measured with DEXA after 7 days of BR (17, 42). Brocca et al. (9) measured fiber CSA of the vastus lateralis after 8 and 35 days of BR and found no change in CSA after 8 days BR but significant reductions after 35 days BR. These findings suggest that longer durations of disuse are necessary to detect atrophy via measurement of vastus lateralis fiber CSA. There are other potential explanations for the discrepancy between DEXA and fiber CSA: *1*) The changes in leg lean mass measured by DEXA after short-term BR may more strongly reflect changes to the lower leg musculature (ie. soleus), which is a postural muscle that is highly susceptible to atrophy during disuse (52); and *2*) a potential redistribution in water compartments inside the fat-free mass. A significant increase in the ratio of extracellular-to-intracellular has been described after weight loss (including fat free mass) (31). Changes in fat free mass assessed by DEXA are also related to a reduction in extracellular fluid and a preservation of intracellular structures and fluid (23). Additionally, Clark et al. (13) found that when compared

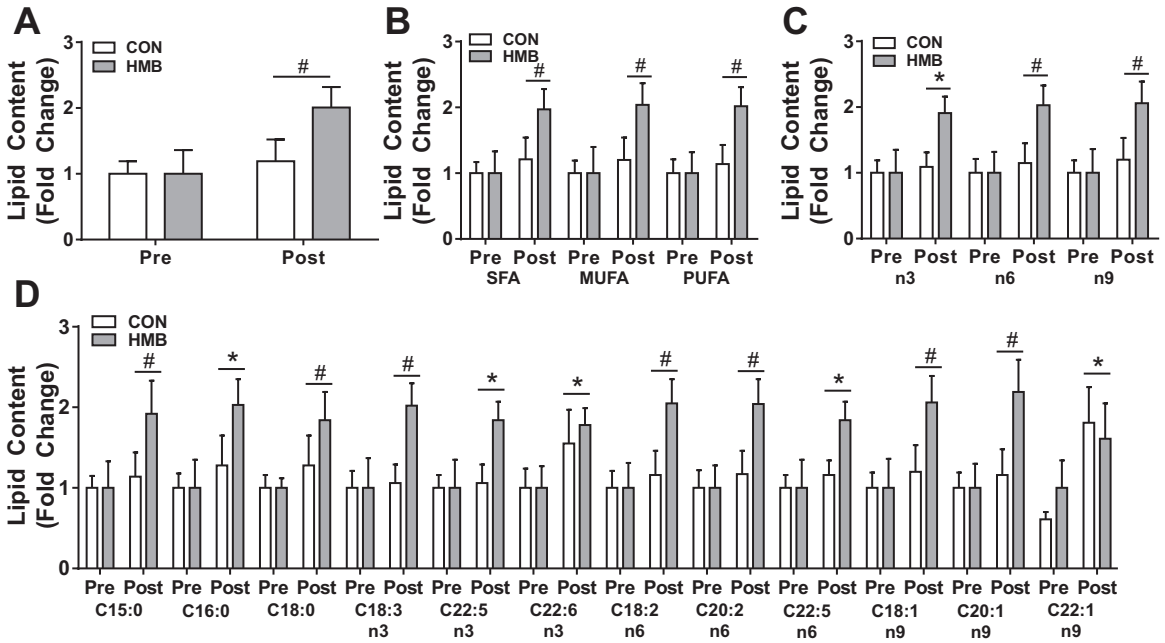

Fig. 4. Skeletal muscle triacylglycerol (TAG) fatty acid content (*A*); total saturated (SFA), monounsaturated (MUFA), and polyunsaturated content (PUFA; *B*); total omega-3 (n3), n6, and n9 fatty acids in TAG (*C*); and various fatty acids present in TAG species (*D*). Pre- and post-BR data are reported as fold change from pre-BR; $n = 4–8$ per group; horizontal bars represent main effects and error bars are SE. #Trend for time effect from pre-BR ($P > 0.05$); *significant main effect for time from pre-BR ($P < 0.05$). See RESULTS for specific P values.

with MRI, DEXA overestimated total lean mass in young and old men and women.

We examined the effects of 10 days of BR on mitochondrial OXPHOS content and dynamics to understand how these processes may be associated with muscle loss during short-term disuse and if HMB may impact these processes. We show that total mitochondrial content remained unchanged following BR in both groups (Fig. 2*A*). These findings are contrast to the findings from studies in younger individuals who had reduced OXPHOS content after 7 days of BR (17). This may be due to muscle age and the activity levels of our subjects before BR. Older or sedentary subjects may need longer periods of disuse to impact their mitochondrial content, which may already be lower compared with younger physically active subjects. We have recently shown that mitochondrial OXPHOS content is similar between young, middle aged, and older sedentary adults (18). However, to our knowledge there are no studies human or animal directly comparing changes in mitochondrial content in young and old after BR or disuse.

We also examined changes in levels of protein associated with mitochondrial dynamics (fusion and fission) and autophagy and total poly-ub proteins as a proxy for the proteasome degradation pathway. We found no changes in levels of proteins involved in mitochondrial dynamics after 10 days of BR (Fig. 3*A*). This was surprising since mitochondrial dysfunction has been proposed as one of the mechanisms leading to muscle atrophy via ROS production (36, 41, 50). Although we did not measure ROS production directly, mitochondrial fission and fusion are thought to help regulate mitochondrial quality, and imbalances in these processes are linked to muscle loss during aging and other atrophy conditions (10, 32). What is more surprising is that both groups did not display any significant changes in proteins involved in mitochondrial content and dynamics, although one group experienced muscle atrophy (control), whereas the other group did not (HMB). This suggests that immobilization-induced muscle atrophy may not have a direct impact on mitochondrial turnover after (10 days of) BR in older adults.

We also found no changes in proteins involved in skeletal muscle autophagy (Fig. 3*B*). These findings are in partial agreement with a recent investigation examining 5 days of BR in young and old participants (51). Tanner et al. (51) found no changes in beclin-1; however autophagasome formation (measured by LC3BII-to-LC3BI ratio) increased in older adults following BR. Although we found no changes in the LC3BII-to-LC3BI ratio in this study, it is possible that there were early changes in these markers in the first few days of BR. It is thought that MPB is the most active during the early stages (1–3 days) of disuse (54); thus, after 10 days, the processes associated with skeletal muscle breakdown and autophagy may have returned to baseline or adaptation to the new level of inactivity was completed. Collectively, these findings highlight the need for studies to investigate the early response to BR.

Interestingly, we found the total amount of poly-ub proteins were elevated in both groups at the end of BR. This suggests muscle is undergoing remodeling after 10 days of BR, regardless of whether it is undergoing atrophy or not. HMB has been shown to lower MPB by inhibiting the ubiquitin-proteasome degradation pathway in various models of muscle atrophy (19, 25, 29, 48, 49). It is possible that HMB may have downregulated the levels or activity of the proteasome leading to degradation of the ubiquitinated proteins. Due to low sample volumes, we were unable to measure other proteins involved in the ubiquitin-proteasome pathway.

Physical inactivity and aging are linked to an increase in fat mass and decrease in muscle mass. HMB has a significant

effect on decreasing fat mass and preserving muscle mass compared with control (1). Oversupply of fatty acids increases fat storage not only in adipocytes but also liver, heart, pancreas, and skeletal muscle (27), which may contribute to muscle atrophy during disuse. While we did not measure plasma free-fatty acids in this study, plasma free-fatty acids have been found to be elevated after 7 days of BR (17) and skeletal muscle intramuscular triglyceride levels to be increased following 28 days of BR under hypercortisolemia (14). Further investigation into the increase in intramuscular triglyceride levels found no increases in diacylglycerols (14) or any other measured lipid species. Fatty acid content in many of the lipid species we measured remained unchanged following BR (Supplemental Tables S1–S11). These findings are in agreement with a 7-day BR study in younger adults (17). Longer durations of disuse may be needed to significantly impact skeletal muscle lipid accumulation (8). Interestingly, we saw a trend for an increase in TAG fatty acid content driven primarily by the HMB group following BR (Fig. 4). These findings suggest a role for HMB to stimulate alterations in fatty acid composition in the TAG pool. A recent investigation found acute HMB supplementation in mouse myotubes increased markers of lipid biosynthesis and elevated total lipid content through the upregulation of peroxisome proliferator-activated receptor-γ and fatty acid synthase (45). These findings may have important metabolic implications as lipids are being stored in the muscle as TAGs and not other bioactive species that are known to impact insulin resistance and inflammation, as seen after a single acute bout of exercise (44).

RT rehabilitation period. We previously reported during the RT rehabilitation following 10 days of BR that the control group regained the leg lean mass lost during BR and the HMB group gaining an additional 0.71 kg over baseline during this period (16). In addition, only the HMB group significantly gained strength above baseline at the end of the rehabilitation period. Following the RT rehabilitation, mitochondrial content remained unchanged or slightly reduced in the CON group while HMB maintained total mitochondrial OXPHOS (Fig. 2*A*). Resistance exercise is widely used to combat disuse atrophy due to its potent anabolic effects resulting in improved skeletal muscle mass and strength. These findings highlight the benefits of combining HMB with RT to help strength recovery via increasing mitochondrial content and are supported by preclinical data showing HMB stimulation of mitochondrial biogenesis and fatty acid oxidation via peroxisome proliferator-activated receptor-γ coactivator-1α stimulation (24). With the increase of mitochondrial OXPHOS in the HMB group, we also found mitochondrial fusion protein MFN2 and fission protein DRP1 were increased in the HMB group (Fig. 3*A*). Future verification is needed using sensitive tools such as imaging methods to directly examine the morphology and number of the mitochondria. Overall, we found little effect of RT rehabilitation on autophagy proteins similar to our findings over the BR period, possibly because this process may occur early during the rehabilitation period. Lastly, the total amount of poly-ub proteins was reduced as a result of the RT rehabilitation in both groups pointing to the benefits of exercise in normalizing muscle protein metabolism.

Limitations and future directions. Our parent study examined the effects of HMB on preserving muscle mass in older adults during extended BR (16). Due to the relatively small sample size in our study, we did not have adequate power to determine significant between-group or gender differences in some variables. We did, however, attempt to infer trends where appropriate, which should be investigated in a larger study. There is a paucity of literature regarding the changes in protein metabolism during the first 1–3 days of BR in older adults. Investigations examining the temporal changes during this period are needed to understand what metabolic pathways that contribute most significantly to muscle atrophy are involved to develop nutritional and other countermeasures for muscle atrophy. Additionally, our studies included healthy older adults and did not have a young group to compare our findings. Future investigations are needed to extend our findings to healthy younger individuals and older adults who may have conditions that influence skeletal muscle mass.

Conclusions and significance. In conclusion, mitochondrial OXPHOS, dynamics, and autophagy were unchanged following 10 days of BR in older adults. HMB stimulated an increase in TAG fatty acid pool, which may protect the muscle from other bioactive lipid species known to stimulate ROS and inflammatory pathways. HMB increased mitochondrial OXPHOS content and dynamics during 8 wk of RT rehabilitation compared with placebo control. Collectively, these findings help explain some of the mechanisms for the benefits of HMB in preserving muscle mass during BR and enhancing muscle strength during exercise recovery. This study highlights a potential new mechanism of action for HMB on skeletal muscle mitochondria when combined with exercise. Additional investigations are needed to further interrogate these mechanisms.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS

R.A.S. and G.D. conceived and designed research; R.A.S. and G.D. performed experiments; R.A.S. analyzed data; R.A.S., G.D., S.L.P., M.T., O.J.K., P.M.C., N.E.D., R.R.W., and B.H.G. interpreted results of experiments; R.A.S. prepared figures; R.A.S. drafted manuscript; R.A.S., G.D., S.L.P., O.J.K., P.M.C., N.E.D., R.R.W., and B.H.G. edited and revised manuscript; R.A.S., G.D., S.L.P., O.J.K., P.M.C., N.E.D., R.R.W., and B.H.G. approved final version of manuscript.

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