METABOLISM CLINICAL AND EXPERIMENTAL 62 (2013) 1718–1729

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Metabolism

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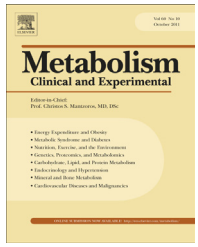

Basic Science

HMB attenuates muscle loss during sustained energy deficit induced by calorie restriction and endurance exercise

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ARTICLE INFO

Article history:
Received 15 January 2013
Accepted 7 June 2013

Keywords:
Mice
Catabolic
Body composition
Functionality
Protein degradation

ABSTRACT

Objective. To investigate the efficacy and underlying mechanisms of β-hydroxy-β-methylbutyrate (HMB) on body composition, muscle mass and physical performance under catabolic versus normal training conditions.

Materials/Methods. Mice were divided into four groups (n = 10/group): 1) ALT = ad libitum + trained (1 h/d for 3 d/wk); 2) ALTH = ALT + HMB (0.5 g/kg BW/d); 3) C = calorie restricted (−30%) + trained (6 h/d, 6 d/wk); and 4) CH = C + HMB. Repeated in vivo assessments included body composition, grip strength and sensorimotor coordination before and after the experimental protocol, while in vitro analyses included muscle wet weights, expression of selected genes and proteins regulating muscle mass, and myofiber cross-sectional area. ANOVAs were used with significance set at $p < 0.05$.

Results. ALTH had greater lean mass than ALT and sensorimotor function increased in ALTH, but decreased in ALT under normal training conditions. Grip strength decreased only in C, but was maintained in CH. Gastrocnemius mass and myofiber CSA were greater in CH than C following catabolic conditions. Gastrocnemius atrogin-1 mRNA expression was elevated in C but not in CH compared to all other groups whereas atrogin-1 protein levels showed no significant changes.

Conclusion. HMB improves body composition and sensorimotor function during normal training and attenuates muscle mass and strength loss during catabolic conditions.

Abbreviations: LBM, lean body mass; SUSOPS, sustained operations; HMB, β-hydroxy-β-methylbutyrate; NHMFL, National High Magnetic Field Laboratory; TB, true baseline; B, baseline; BH, baseline + HMB; ALT, ad libitum-trained; ALTH, ad libitum-trained + HMB; C, caloric restricted + trained; CH, caloric restricted + trained + HMB; DXA, Dual Energy X-ray Absorptiometry; CSA, cross-sectional area; GAPDH, glyceraldehydes 3-phosphate dehydrogenase; RT-PCR, Reverse Transcription Polymerase Chain Reaction.

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http://dx.doi.org/10.1016/j.metabol.2013.06.005

1. Introduction

Operations conducted by tactical personnel (e.g. military, police, fire/rescue and emergency services) are often complicated by sustained high-energy expenditure, low caloric intakes, sleep deprivation and environmental exposures to heat, cold and/or altitude. The loss of lean body mass (LBM) occurs during both short-term [1] and long-term operational stress [2], and lower-body anaerobic power is adversely affected by short-term operational stress [1]. It is evident that caloric restriction combined with continuous physical activity creates a catabolic milieu that causes loss of LBM and performance decrements [1,2]. It is important to understand the underlying mechanisms of this unique catabolic condition and identify appropriate countermeasures to attenuate the loss of muscle in tactical personnel during sustained operations (SUSOPS). β-hydroxy-β-methylbutyrate (HMB) is a metabolite of the branched-chain amino acid leucine and is produced endogenously in small amounts. HMB promotes gains in strength and LBM during resistance training [3] and attenuates muscle loss during pathological conditions (i.e. muscular dystrophies, trauma and cancer cachexia) [4]. HMB attenuates muscle loss by inhibiting protein degradation and stimulating protein synthesis [5]. The primary mechanism of HMB action seems upregulate the mTOR/p70S6K signaling pathway which leads to protein synthesis and muscle hypertrophy [6]. The benefits of HMB on muscle cell proliferation, fusion, and cell survival [7], together with its previously demonstrated anti-catabolic effects [5] justify this supplement as a therapeutic agent to prevent muscle loss in myopathies as well as in aging, trauma, and cancer cachexia. However, information is lacking concerning the efficacy of HMB on skeletal muscle in a catabolic state induced by a combination of sustained physical activity and low calorie intake. The purpose of this study was to investigate the efficacy and underlying mechanisms of HMB on LBM, muscle mass and physical performance under normal training conditions with ad libitum diet versus catabolic conditions induced by prolonged endurance exercise combined with caloric restriction. Our a priori hypotheses were that HMB would enhance muscle mass and physical performance under normal training conditions and it would attenuate the loss of muscle mass and physical performance under catabolic conditions.

2. Methods

2.1. Animals and experimental design

All experimental procedures were approved by the Animal Care and Use Committee of The Florida State University, Tallahassee, Florida. This procedure is in accordance with the policy statement of the American College of Sports Medicine on research with experimental animals. Sixty-one, six-week old C57BL/6 male mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Upon receipt, mice were housed singly in an animal housing facility at the National High Magnetic Field Laboratory (NHMFL) located at FSU. Mice were acclimatized for three days and then randomized into three baseline groups: 1) TB = true baseline, sedentary control (n = 7); 2) B = baseline (n = 27); and 3) BH = baseline + HMB (0.5 g/kg BW/d) (n = 27). The B and BH groups underwent a four-week run-in phase to simulate initial entry training that soldiers go through upon entering the military where mice exercised three days a week for one hour each day at a speed of 6 m/min (i.e. fast walk) on a forced exercise wheel. After this four-week phase, a cohort of mice (n = 7) from each of the three groups was sacrificed for baseline tissue isolation of the gastrocnemius and soleus muscles. The remaining mice (n = 40) were randomly assigned from the B group into ALT and C groups and from the BH group into ALTH and CH groups (n = 10/group): 1) ALT = ad libitum-trained (exercised 1 h/d for 3 d/wk, 6 m/min); 2) ALTH = ad libitum-trained + HMB (0.5 g/kg BW/d); 3) C = caloric restricted (−30% of ad libitum groups) + trained (~6 h/d = 2 km/d, 6 d/wk, 6 m/min speed); and 4) CH = C + HMB. The ad libitum-trained groups represented normal training conditions with or without HMB, while caloric restriction combined with prolonged endurance exercise (i.e., C and CH) represented the catabolic condition groups with or without HMB. The ALT and ALTH groups continued the same training regimen (exercised 1 h/d for 3 d/wk, 6 m/min) as during the four-week initial training phase in order to simulate the normal training all soldiers undergo when not in a sustained operation, whereas the C and CH groups simulated a six-week sustained operation based on previous research demonstrating loss of LBM in mice [8] and on evidence that soldiers can be on sustained operations for at least one month or longer. A flowchart of the experimental design is provided in Fig. 1. This combination of calorie restriction and prolonged endurance exercise in the mice was meant to mimic the 40%–45% energy deficit inherent in soldiers undergoing sustained operations. This six-week catabolic period consisted of cycles comprised of six days of calorie restriction and prolonged fast walking on an exercise wheel (2 km/d, ~ 6 h/d, 6 m/min) followed by a 24-h recovery period. The forced exercise wheel (Model 80800A, Lafayette Instrument, Lafayette, IN, USA) used in the present study provided a novel paradigm given that previous research primarily used voluntary wheel running [8]. In addition, the forced exercise provided relevance to military training/missions along with precise control of activity level. The 24-h recovery period each week consisted of no exercise, and the catabolic groups were provided the average daily amount of feed that was consumed by the normal training groups. Tissue isolation was repeated in all remaining mice after the six-week experimental protocol and compared with baseline samples. Baseline measurements of body composition (Dual Energy X-ray Absorptiometry, DXA), strength (grip strength) and sensorimotor function (incline plane test) were conducted after the initial four-week phase and repeated after the six-week experimental protocol. Tissue collection was conducted at the NHMFL prior to (baseline groups) and after the six-week experimental protocol. All mice were kept on a reverse 12-h dark/light cycle and were provided with free access to acidified distilled water. Mice were monitored daily for physical signs of distress.

2.2. HMB administration and diet

Average daily food consumption values for adult mice typically range from 3.5 to 4 g/d for a 25 g mouse. Based on their average

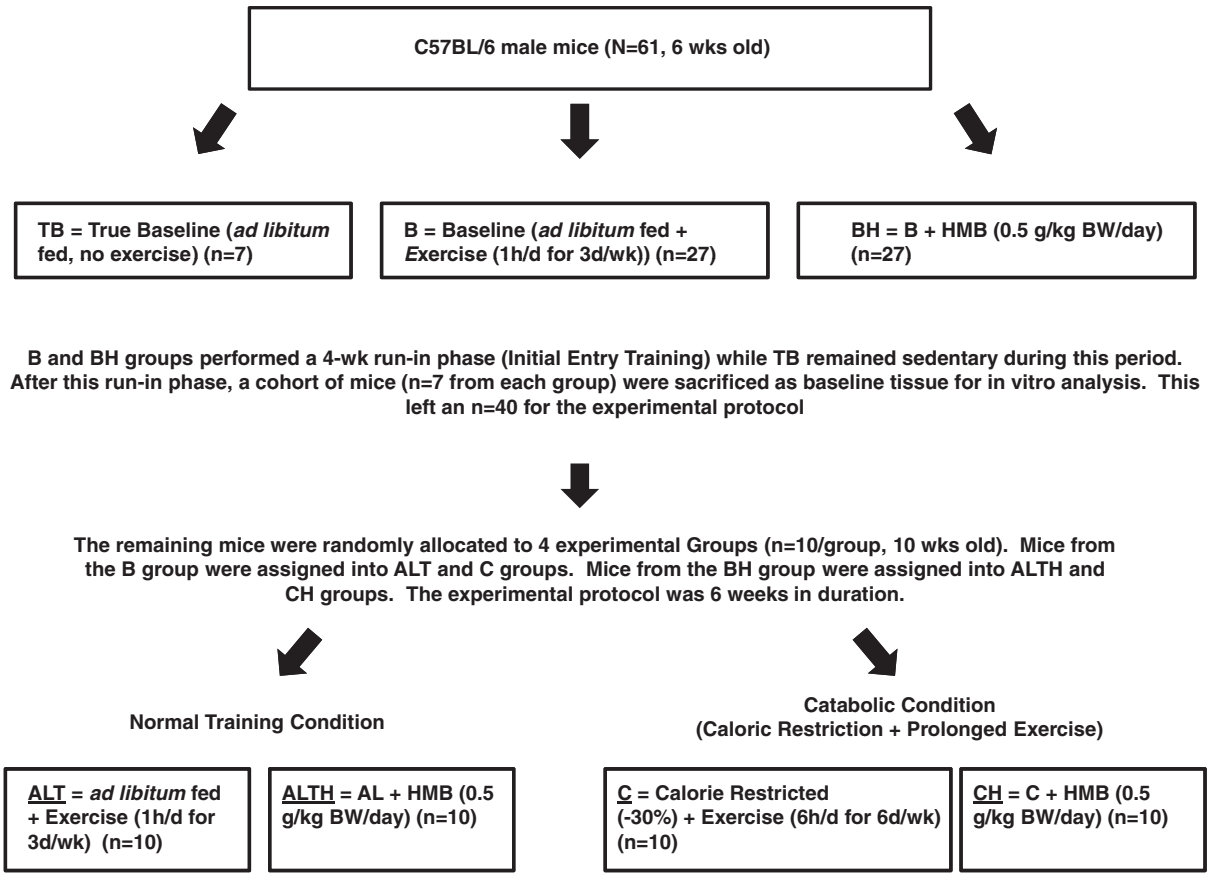

Fig. 1 – Flowchart of the experimental design.

diet, the HMB dosage was calculated as approximately 1% (w/w) calcium HMB to achieve about 0.5 g HMB/kg BW/d dose [9]. This dose of HMB was chosen based on previous human studies (6 g/d) and because this dose was used previously to investigate HMB supplementation in rodent models from our laboratory [10]. We calculated a human-to-rodent conversion based on the assumption that a rodent's metabolic rate per kg body weight is at least six times greater than humans [11]. Therefore, in order to provide an appropriate and safe dose for each animal, the conversion was calculated as follows: (6 g/75 kg BW) × 6 = 0.5 g/kg BW/d HMB [9]. HMB was professionally mixed in semi-purified pellet animal chow by Research Diets (New Brunswick, NJ, USA). The HMB groups were administered with HMB in their food starting at the beginning of the initial four-week phase and throughout the six-week experimental period.

The normal training groups consumed an AIN-76A diet (Research Diets, New Brunswick, NJ, USA) [8] with or without HMB. The catabolic condition groups consumed 30% fewer calories than the normal training groups. The mass of pellet consumed was measured on a daily basis by weighing the food remaining in the normal training groups and subtracting it from the food that was administered the day before. This mass was then multiplied by 0.71 to determine the amount of daily food provided to the catabolic condition groups. Therefore, when feeding 30% less calories you are feeding 30% less carbohydrate but the same g level of protein, vitamins, minerals, and fiber as the ad libitum fed animals. Table 1 provides an estimated composition of the experimental diets.

2.3. *Body composition using Dual Energy X-ray Absorptiometry (DXA)*

Mice were anaesthetized, and their whole body was measured in vivo by means of dual energy X-ray absorptiometry (iDXA; GE Medical Systems, Madison, WI, USA) to determine total body mass, lean body mass and fat mass. Baseline DXA

Table 1 – Diet composition.

Ingredient	AIN-76A	30% CR	AIN-76A + HMB	30% CR + HMB
	g			
Casein	200	200	200	200
DL-Methionine	3	3	3	3
Corn Starch	150	82.3	150	82.3
Sucrose	500	275	500	275
Cellulose, BW200	50	50	50	50
Corn Oil	50	50	50	50
Mineral mix	35	35	35	35
Vitamin mix	10	10	10	10
Choline bitartrate	2	2	2	2
HMB	0	0	3.584	3.584
HMB, dose/kg bodyweight	0	0	0.50	0.50

AIN-76A Rodent Diet and 30% Caloric Restriction Diet with or without HMB (Provides 0.5 g/kg BW/d HMB assuming 25 g mouse and 3.5 g/d feed intake).

measurements were performed after the four-week run-in phase and again after the six-week experimental protocol. The CV for DXA lean tissue mass was 8.3%.

2.4. Muscle strength (Grip strength) test

The purpose of this test was to evaluate the strength of the animal's forelimb muscles [12,13] before and after the six-week experimental period. In the grip strength test, the animal's forelimbs were placed on a tension bar while it was restrained manually by the scruff of the neck and base of the tail with a towel placed over the animal. The animal was gently pulled back until it lost its grip from the bar. The force generated as it attempted to maintain its grip was measured in grams by a strain gauge (DFS-101 Force gauge, AMETEK TCI, CA, USA). Each animal was subjected to three trials with the greatest force of the three trials being the criterion measure. The CV for the grip strength test was 4.2%.

2.5. Sensorimotor coordination (Inclined plane) test

The purpose of this test was to evaluate sensorimotor coordination before and after the six-week experimental period. The inclined plane test was adapted from Murphy et al. [14]. Each animal was placed onto the surface of a rectangular Plexiglas® plane (60 × 122 cm) inclined at a fixed angle (beginning at 50°). Animals were placed facing the upper edge of the plane at 10 cm from the top and were released after a 5-s delay allowing for stable footing. If the animal did not freely slide backward within 5 s, the trial was scored as a success. The angle of inclination then was increased or decreased by 2°, and the procedure repeated following a rest period of at least 5 min. When the mouse was unable to remain in position for 5 s despite three trials, it was determined that the mouse had failed at the angle, and the angle prior to failure was recorded by the investigator. The CV for the sensorimotor coordination test was 3.8%.

2.6. Tissue collection: muscle isolation

Muscle samples were collected at the NHMFL using surgical methods prior to (baseline groups) and after the six-week experimental protocol. After the mice were euthanized using a carbon dioxide gas overdose, the gastrocnemius and soleus muscles were isolated. Tissue samples were immediately weighed, divided and snap frozen (30–35 mg per tube).

2.7. Morphological analysis for gastrocnemius CSA

For tissue sectioning, frozen, transversely mounted gastrocnemius was positioned in a −20 °C cryostat (ThermoScientific, Dubuque, IA) and cut into serial cross-sections at 7 μm trim thickness. Three cross-section trims for each muscle sample were placed on each well of a three-well slide. Each section was fixed than incubated for 1 min in two drops of hematoxylin at room temperature. Myofibers were stained light blue–purple and myonuclei stained dark blue. After staining, sections were washed in nano-pure water and rinsed in PBS. Slides were then mounted in Vectamount medium (Vector Laboratories, Burlingame, CA) and dried before microscopy. Gastrocnemius cross-sections were visualized and imaged utilizing acquisition software for microscopy (AxioVision 4.8, Carl Zeiss Microimaging, Thornwood, NY) at an objective magnification of 20×. For analysis and measurement of CSA, cell membrane perimeters were traced and quantified using Axio Vision Rel. 4.6 (ZEISS). One image per sample was analyzed. The total average area within the membrane outline was used to determine the cross-sectional area per μm^2.

2.8. Western immunoblot analysis for gastrocnemius

Gastrocnemius muscles were homogenized in ice-cold lysis buffer containing 50 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1% Triton X-100, 1 mmol/L PMSF, and complete protease inhibitor cocktail. Gastrocnemius extracts were centrifuged at 13,000 rpm for 15 min at 4 °C. Next, the protein in the supernatant was quantified using the BCA method. Sixty micrograms of total protein was resolved on 12% SDS-PAGE gel (150 V, 25 °C, 2 h) and then transferred to PVDF membranes (12 V, 25 °C, 1 h). All of the blots were then incubated with Ponceau S (Sigma, USA) to ensure equal loading in all lanes (data not shown). For the detection of atrogin-1 and myogenin, the membranes were probed with atrogin-1 (sc-33782, 1: 1000) and myogenin (sc-578, 1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA), antibodies overnight at 4 °C in 5% BSA in Tris-buffered saline (TBS) with 0.05% Tween 20. The membranes were then washed three times for 5 min each in 0.1% TBST, after which they were incubated for 90 min with appropriate secondary antibody (1:5000). The membrane was then washed as described above, after which enhanced chemiluminescent (ECL) reagent (GE Healthcare, UK) was applied according to the manufacturer's instruction. Target protein signals were visualized by Bio-Rad ChemiDoc™ XRS imaging system and quantified by densitometry using Bio-Rad QuantityOne® software (Bio-Rad Laboratories, Hercules, CA, USA). Target protein levels were then normalized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as in previous studies [15–21].

2.9. Semi-quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR)

As described in previous studies [22], a semi-quantitative RT-PCR method using 18s ribosomal RNA as an internal standard was used to determine relative expression levels of target mRNAs regulating muscle mass. To assess the regenerative capacity, we analyzed muscle mRNA expressions of myogenic MyoD (forward 5′-CGCCGCCGCCTGAGCAAAGT-3′ and reverse 5′-GGGCGCGGCGTCCTGGTC-3′); and myogenin (forward 5′-CAGGAGCCCCACTT CTATGATG-3′ and reverse 5′-CACT-GGCCTGGACAATGCTC-3′], mitogenic [IGF-IEa (forward 5′-ATCTGCCTCTGTGACTTCTTGA-3′ and reverse 5′-CTGGAG-CCATAGCCTGTG-3′); and IGF-IEb (or MGF: forward 5′-TCCTTATGAATTGGCTTATC-3′ and reverse 5′-GTTTGTCA-TCTTCCATTCTGTT- 3′ or mechano-growth factor)] regulatory factors, and myostatin (forward 5′-ACAATCTAGGCAAG-GAAGTGAG-3′ and reverse 5′-GAATGGCAATGTGTATG-TAAGG-3′), and its receptor, activin type IIB receptor (ActRIIB: forward 5′-ACCCGGCATGAAGCACGAAAAC-3′ and reverse 5′-GGCCCTCACCACGACACCAC-3′). We assessed protein synthesis and protein degradation by measuring

expressions of protein kinase B (Akt: forward 5′-CGGCCACGC-TACTTCCTCCTC-3′ and reverse 5′-GCCCATTCTTCCC-GCTCCTCAG-3′), and mammalian target of rapamycin (mTOR: forward 5′-GCCCACGCCTGCCATACTTG-3′ and reverse 5′-TCAGCTCCGGGTCTTCCTTGTT-3′), and atrogin-1 (forward 5′-CGTGCACGGCCAACAACC-3′ and reverse 5′-CCCGCCAACGTCTCCTCAAT-3′), and muscle ring finger-1 (MuRF1: forward 5′-GGCTGCGAATCCCTACTGG-3′ and reverse 5′-TGATCTTCTCGTCTTCGTGTTCCT-3), respectively. DNAStar Lasergene 7 (Madison, WI) software was utilized to design each set of forward and reverse primers, and all primer sets were tested for optimal conditions. The procedure of RNA isolation has been detailed previously [23]. For mRNA studies, 30 mg of muscle yielded approximately 6–12 μg of RNA. As described, 1 μg of RNA is reverse transcribed in a total volume of 20 μL using SuperScript II Reverse Transcriptase with a mix of oligo (dT) (Invitrogen, Carlsbad, CA). A mix of oligo (dT) (100 ng/reaction) and random primers (200 ng/reaction) was utilized. After 50-min incubation at 45 °C, the RT reaction mixtures were heated at 90 °C for 5 min to discontinue the reaction, and cDNA then was stored at −80 °C for subsequent PCR analyses. Each PCR co-amplified 18s (324-bp product), with each target mRNA to express each as a ratio of target mRNA/18 s. Ethidium bromide (0.1 μg/mL) was pre-mixed in the gel, and images were captured under UV and analyzed via a Bio-Rad ChemiDoc™ XRS imaging system and a Bio-Rad QuantityOne® software (Bio-Rad Laboratories, Hercules, CA, USA).

2.10. Statistical analysis

Statistical analysis was performed by Statistica (Statsoft, Tulsa, OK). A 4 (group) × 2 (time) repeated measures analysis of variance (ANOVA) was used for in vivo measures including body composition, grip strength and sensoriomotor coordination assessments. A one-way ANOVA was used to analyze in vitro variables including tissue wet weights, mRNA expression, protein expression and myofiber CSA. Fisher's least significant difference (LSD) test [24] was used for post-hoc analysis to localize main or interaction effects. For our sample size, we were primarily interested in the interaction effect from a group × time ANOVA. Briefly, relevant data (means ± SD) involving the effects of energy restriction and exercise on lean body mass were assembled from a recent publication and used to calculate sample size [8]. With 10 mice per group (total n = 40), our study achieved 80% power to detect a statistically significant ($p < 0.05$) group × time interaction assuming LBM 16.1 ± 1.4 g at study inception and LBM of 13.8 ± 1.0 g at the end of the study. We also figured in a 40% dropout rate in mice to give us an n = 10 per group. All data are presented as means ± standard error (SE). Significance was set at $p < 0.05$.

3. Results

3.1. Body composition

3.1.1. Total body mass

There was a significant group × time interaction for total body mass (TBM). TBM increased in ALT (+8%) and ALTH (+7%), and decreased in C (−23%) and CH (−18%) after the six-week experimental period. Both catabolic groups had lower TBM than both normal training groups after the six-week protocol. There was no difference in TBM between ALT, and ALTH, but CH had greater TBM (+8%) than C after the experimental protocol (Table 2).

3.1.2. Lean body mass

There was a significant group × time interaction for lean body mass (LBM). The normal training groups had greater LBM than both catabolic groups after the experimental protocol. The ALTH group had greater LBM (+17%) than ALT, and the C group had greater LBM (+17%) than CH after the experimental protocol (Table 2).

3.1.3. Fat mass

There was a significant group × time interaction for fat mass (FM). FM increased in ALT (+25%) and the ALTH group had less FM (−12%) than ALT after the protocol. Both catabolic groups had lower FM than both normal training groups after the protocol. FM decreased in C (−56%) and CH (−38%) after the protocol, and C had lower FM (−31%) than CH after training (Table 2).

3.2. Muscle strength (Grip Strength)

There was a significant group × time interaction for grip strength. There was an increase in both ALT (+7%) and ALTH (+12%) after the experimental protocol. The C group decreased (−10%) grip strength whereas CH attenuated this loss after the protocol. Additionally, CH had greater grip strength (+11%) than C after the protocol (Table 2).

3.3. Sensorimotor function (Incline Plane Test)

There was a significant group × time interaction for sensorimotor function. The ALT group decreased (−8%) while ALTH increased (+7%) after the protocol. There were a decrease in CH (−12%) and a trend for a decrease in C ($p = 0.057$, −6%) after the catabolic conditions. The ALTH group was greater (+10%) than ALT after the protocol (Table 2).

3.4. Tissue mass: muscle wet weight

There was a significant group effect for gastrocnemius muscle wet weight. Both catabolic groups exhibited significantly lower gastrocnemius mass than both normal training groups (ALT and ALTH) after the protocol. Gastrocnemius mass in CH group was significantly greater (+10%) than C group after the protocol. There was no difference in soleus mass between groups (Table 3).

3.5. Gastrocnemius myofiber Cross-Sectional Area (CSA)

Gastrocnemius cross-sectional images are shown in Fig. 2A. There was a significant group effect for gastrocnemius myofiber cross-sectional area (CSA). The mean CSA (μm^2) was 38% lower in C compared to the ad libitum normal training groups after the experimental protocol. The mean CSA (μm^2) of C (1312 ± 143 μm^2) was lower (−35%) compared to CH (2015 ± 193 μm^2) after the

Table 2 – In vivo measures of body composition and functional measures pre- and post-experiment.

	ALT		ALTH		C		CH	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post
DXA Measures								
TBM (g)	24.2 ± 1.9	26.2 ± 2.0 *	24.6 ± 1.4	26.3 ± 2.3 *	24.8 ± 1.9	19.1 ± 0.8 *,!	25 ± 1.3	20.6 ± 0.7 *,!
LBM (g)	9.1 ± 1.2	8.7 ± 2.1	9.7 ± 1.5	10.2 ± 1.0 @	8.1 ± 1.0	7.9 ± 1.6 !	8.5 ± 1.4	6.5 ± 1.4 *,!,#
FM (g)	10.9 ± 1.1	13.6 ± 2.4 *	11.2 ± 1.6	12.1 ± 2.0 @	12.7 ± 1.7	5.6 ± 1.4 *,!	13.1 ± 1.4	8.1 ± 1.4 *,!,#
Functional Measures								
Grip Strength (g)	192.2 ± 11.8	205.7 ± 21.5 *	195.3 ± 15.7	217.9 ± 26.1 *	193.3 ± 11.9	173.9 ± 6.3 *	194.3 ± 11.1	192.6 ± 10.5
Incline Plane (°)	36.6 ± 2.3	33.6 ± 3.1 *	34.6 ± 3.1	37.0 ± 2.4 *	34.8 ± 3.0	32.7 ± 1.6	35.8 ± 2.6	31.6 ± 2.1 *

ALT = ad libitum-trained (exercised 1 h/d for 3 d/wk, 6 m/min); ALTH = ALT + HMB (0.5 g/kg BW/d); C = caloric restricted (−30% of ad libitum groups) + exercise (~6 h/d = 2 km/d, 6 d/wk, 6 m/min speed); and CH = C + HMB (0.5 g/kg BW/d); DXA (Dual Energy X-ray Absorptiometry); TBM(Total Body Mass); LBM(Lean Body Mass); FM(Fat Mass); Data are presented as mean ± SE.
* $p < 0.05$, significantly different from baseline group.
@ $p < 0.05$, significantly different between ad libitum groups.
# $p < 0.05$, significantly different between catabolic groups.
! $p < 0.05$, significantly different between catabolic and ad libitum groups.

experimental protocol (Fig. 2B). CH significantly attenuated the decrease in fiber CSA of the gastrocnemius.

3.6. *Muscle mass regulators*

3.6.1. *Expression of myofiber size regulating proteins in gastrocnemius*

There were no group differences for protein concentration (μg/μl, data not shown) and protein expression of atrogin-1 (Fig. 3A) and myogenin (Fig. 3B) in the gastrocnemius.

3.6.1.1. Regulators of myogenesis and mitogenesis. MyoD was analyzed as an early regulator of satellite cell differentiation. There were no significant group effects in the gastrocnemius or soleus for MyoD mRNA levels (data not shown). Myogenin was analyzed as a late regulator of satellite cell differentiation. In the gastrocnemius, myogenin mRNA was greater in both catabolic groups than all baseline and both normal training groups. There was no difference in myogenin expression between the catabolic groups (Fig. 3D). Myostatin and ActRIIB were analyzed as an inhibitor of muscle differentiation and growth. There were no significant group effects in the gastrocnemius or soleus for ActRIIB. There was no significant group effect for myostatin mRNA in the soleus or gastrocnemius (data are not shown). Insulin-like growth factor-I (IGF-IEa and IGF-IEb or mechano-growth factor, MGF) was analyzed as a positive regulator of mitogenesis [25]. There were no significant group effects for any of the muscles analyzed (data not shown).

3.6.1.2. Regulators of protein synthesis. Protein kinase B (Akt) and mammalian target of rapamycin (mTOR) were analyzed as key markers for protein synthesis [26]. There was a significant group effect for Akt mRNA in the gastrocnemius and soleus muscle. Akt mRNA in the gastrocnemius was greater in C group than all baseline groups. Akt mRNA was greater in both the C and CH groups than both normal training groups after the protocol (Fig. 4D). In soleus muscle, Akt mRNA only in C group was greater than B, BH and both normal training groups (Fig. 4E).

There was a significant group effect for soleus, but not gastrocnemius for mTOR mRNA (Fig. 4F). In soleus, only C was greater than all baseline groups and both normal training groups (Fig. 4G).

3.6.1.3. Regulators of protein degradation. Atrogin-1 and MuRF1 were analyzed as markers of protein degradation and ubiquitin ligase activity [27]. There was a significant group effect for soleus, but not gastrocnemius for atrogin-1 mRNA (Fig. 3C). Atrogin-1 mRNA in the soleus of the C group was greater than all baseline and both normal training groups after the protocol. The CH group was greater than B, BH, and both normal training groups (Fig. 4A).

There was a significant group effect for the gastrocnemius and soleus for MuRF1 mRNA. In gastrocnemius, C group MuRF1 mRNA was greater than TB, BH and both normal training groups after the protocol. The CH group was greater than TB and both normal training groups. There was no

Table 3 – In vitro measures of tissue weights for studied groups.

	TB	B	BH	ALT	ALTH	C	CH
Gastrocnemius (mg)	148 ± 9.0	137.6 ± 6.4 %	144.1 ± 14.5	141.7 ± 9.5	142.9 ± 5.5	102.5 ± 8.4 *,!	113.8 ± 9.9 *,!,#
Soleus (mg)	8.8 ± 1.4	7.3 ± 2.2	8 ± 2.6	7.6 ± 2.0	7.6 ± 3.2	7.4 ± 0.8	6.9 ± 2.0

TB = True Baseline; B = Baseline; BH = Baseline + HMB; ALT = ad libitum-trained (exercised 1 h/d for 3 d/wk, 6 m/min); ALTH = ALT + HMB (0.5 g/kg BW/d); C = caloric restricted (−30% of ad libitum groups) + exercise (~6 h/d = 2 km/d, 6 d/wk, 6 m/min speed); and CH = C + HMB (0.5 g/kg BW/d); Data are presented as mean ± SE.
% $p < 0.05$, significantly different from TB group.
* $p < 0.05$, significantly different from baseline group.
# $p < 0.05$, significantly different between catabolic groups.
! $p < 0.05$, significantly different between catabolic and ad libitum groups.

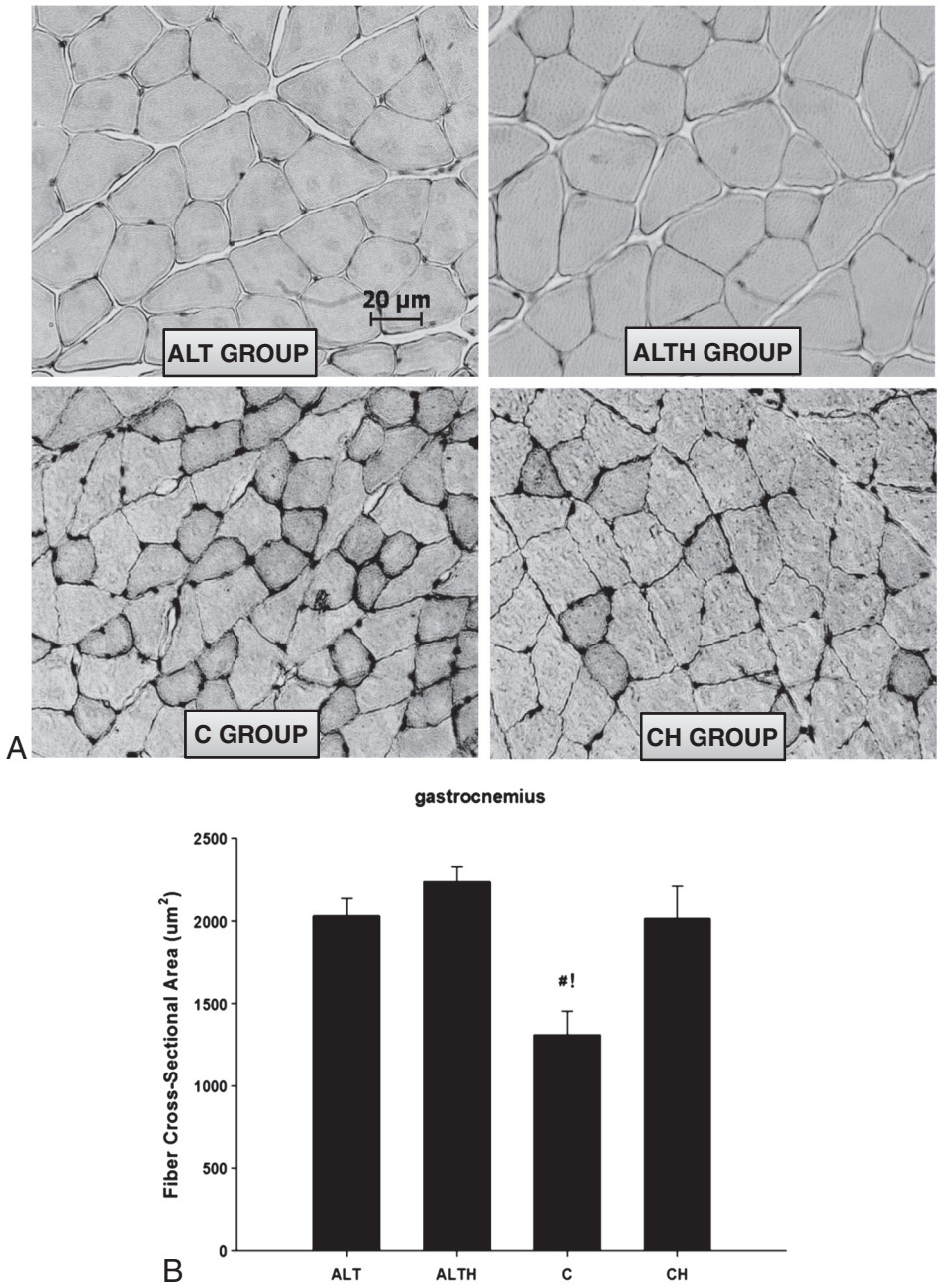

Fig. 2 – Image of gastrocnemius muscle fiber cross-sectional area (A) and quantification of gastrocnemius muscle fiber cross-sectional area (B). Values are means ± standard error. ! $p < 0.05$, significantly different between catabolic and ad libitum groups. # $p < 0.05$, significantly different between catabolic groups.

difference between the C and CH groups (Fig. 4B). In soleus, only C group MuRF1 mRNA was greater than all baseline groups and both normal training groups. The C group was also greater than the CH group after the protocol (Fig. 4C).

4. Discussion

The overarching aim of the present study was to investigate the efficacy and underlying mechanisms of HMB on LBM, muscle mass and physical performance under normal conditions with ad libitum diet versus catabolic conditions induced by prolonged endurance exercise combined with caloric restriction. The major findings of the study were: 1) HMB intake during a catabolic condition attenuates loss of strength, gastrocnemius mass and myofiber CSA, but not lean mass (DXA) and 2) HMB increased LBM, attenuated increases in fat mass and improved sensorimotor function under normal training conditions.

HMB intake attenuates the loss of gastrocnemius mass and myofiber CSA during catabolic conditions imposed by calorie restriction and prolonged endurance exercise. Loss of gastrocnemius mass in our catabolic mice agrees with Katzeff and colleagues [28] who demonstrated significantly less muscle

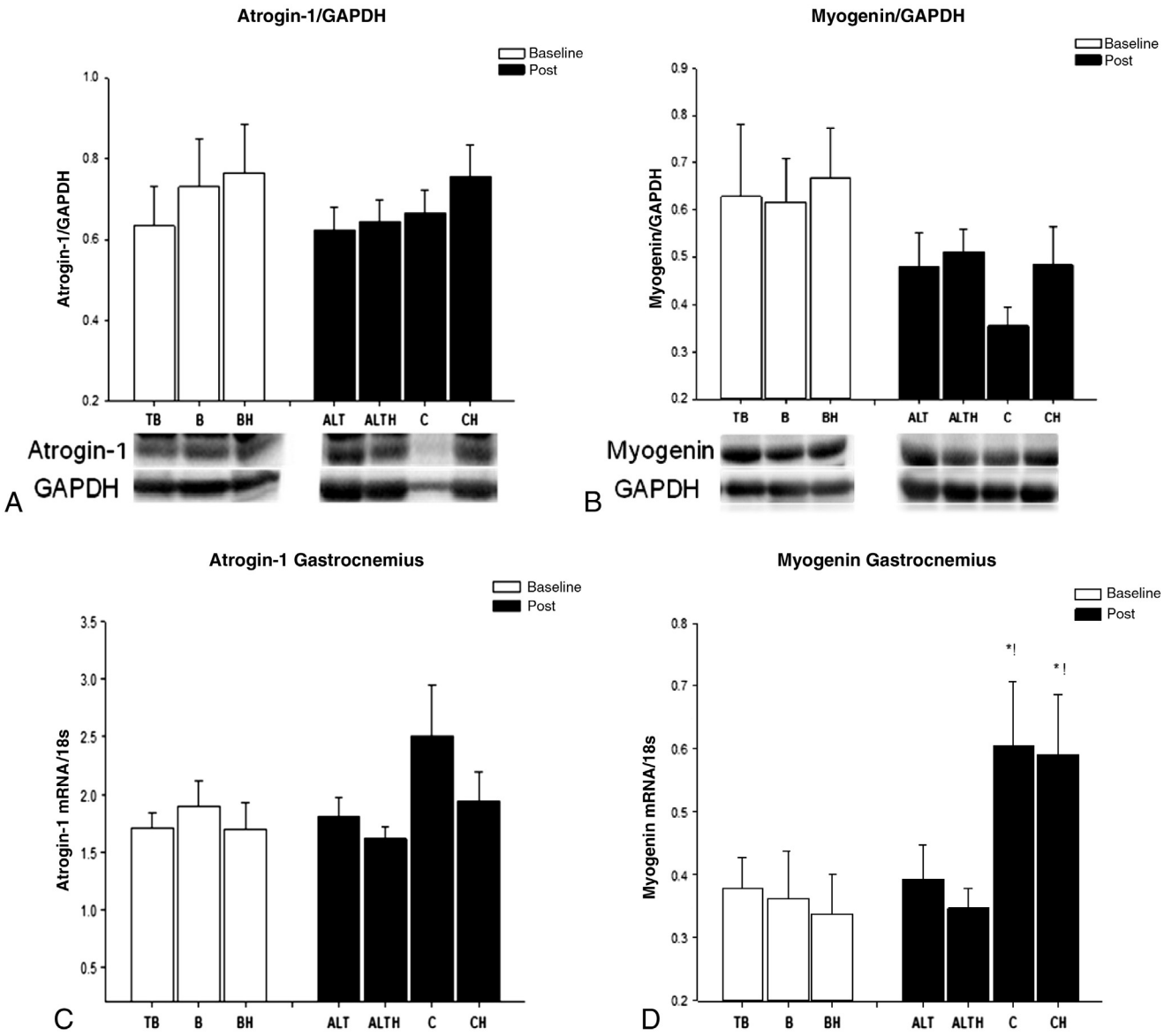

Fig. 3 – Protein levels of atrogin-1 (A) and myogenin (B) in the gastrocnemius. mRNA expression of atrogin-1 (C) and myogenin (D) in the gastrocnemius. Values are means ± standard error. * $p < 0.05$, significantly different from all baseline groups. ! $p < 0.05$, significantly different between catabolic and ad libitum groups.

mass in rats following four weeks of caloric restriction (−25%) and voluntary wheel running (~4 km/d). Our histology data demonstrated that consuming HMB under these catabolic conditions attenuated loss of gastrocnemius mass and gastrocnemius fiber CSA. This finding agrees with Smith et al. [5] who also demonstrated an increase in muscle mass in cancerous mice (MAC 16 mouse model) following HMB consumption. Soleus mass was not significantly altered in the catabolic group mice which may be due to the fact that the soleus is a red oxidative postural muscle consisting mainly of type I muscle fibers that are stimulated by normal daily activities and endurance exercise. Given that the exercise protocol consisted only of endurance exercise; this may have attenuated the significant decrease in soleus mass in the catabolic group mice.

Our findings demonstrated that HMB attenuated the loss of strength under catabolic conditions. As anticipated, catabolic mice demonstrated a significant decrease in grip strength whereas HMB consumption attenuated this decrease. Although we didn't measure forelimb muscle mass; the characteristics of our wheel exercise training model requires all four limbs; therefore the maintenance of gastrocnemius mass may be generalized to other limb muscles and demonstrates an ergogenic effect of HMB under these catabolic conditions. This finding agreed with previous findings in our laboratory demonstrating that HMB administration enhanced muscle strength during resistance training in aged (22 months) female rats [10].

HMB enhanced body composition and sensorimotor function under normal training and eating conditions. LBM improved (+17%) and fat mass was significantly less (−12%) in normal condition mice consuming HMB than in mice not consuming HMB even though these groups only exercised aerobically three days/week for one hour each day. This

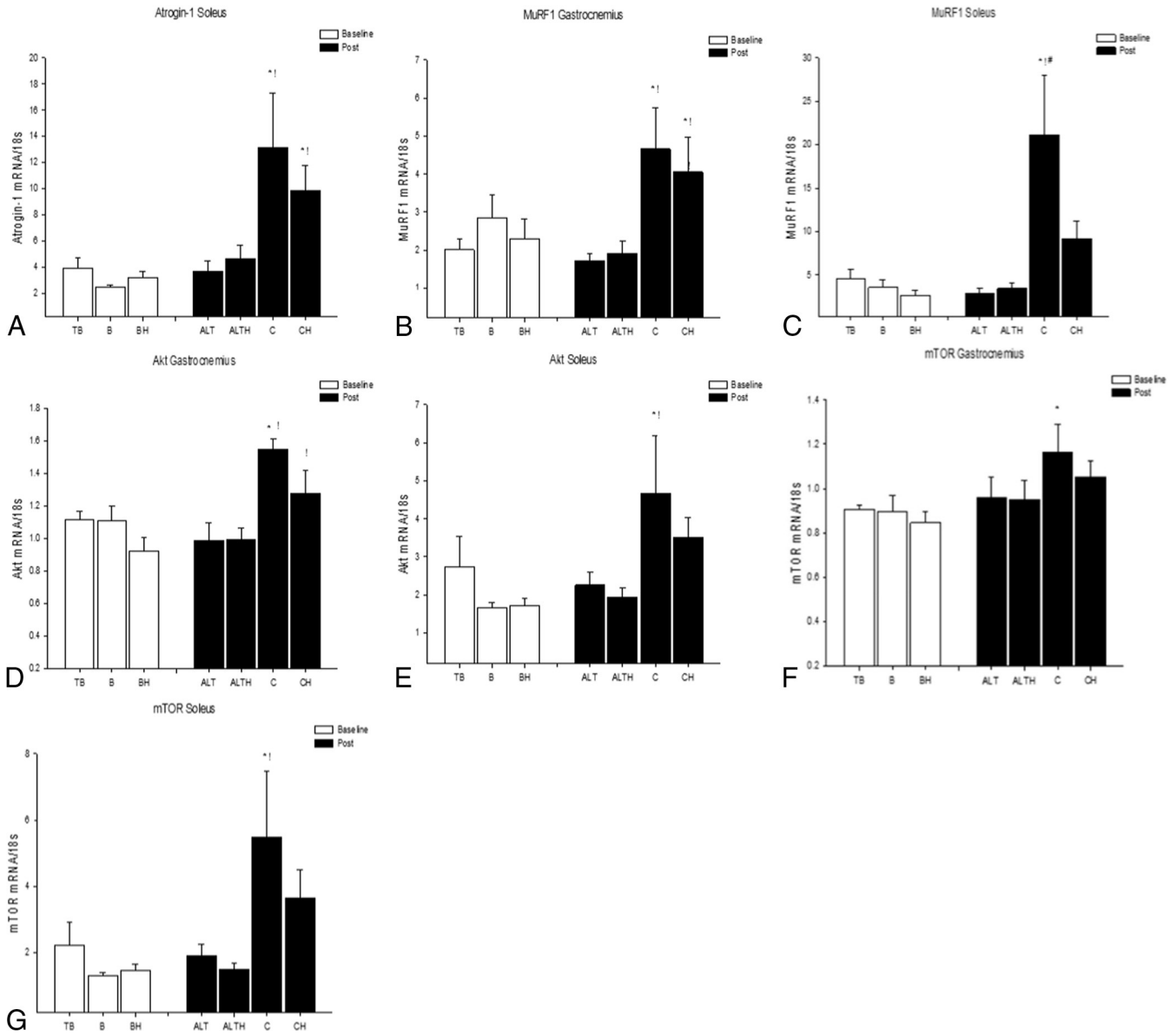

Fig. 4 – Atogin-1 mRNA expression in the soleus (A), MuRF1 mRNA in the gastrocnemius (B), and soleus (C), Akt mRNA expression in the gastrocnemius (D), and soleus (E), mTOR mRNA expression in the gastrocnemius (F), and soleus (G). Values are means ± standard error. * $p < 0.05$, significantly different from all baseline groups. ! $p < 0.05$, significantly different between catabolic and ad libitum groups. # $p < 0.05$, significantly different between catabolic groups.

finding agrees with previous research demonstrating greater fat loss following HMB administration [3]. The lower LBM in the normal condition group not consuming HMB compared to normal condition consuming HMB after the protocol may explain the decrement in sensorimotor function seen in normal condition mice not consuming HMB. Specifically, hindlimb strength is a crucial factor in a mouse's ability to maintain an isometric contraction in order to maintain its bodyweight on an incline plane [14]. Sensorimotor function was enhanced only in normal condition mice consuming HMB, suggesting that HMB may be beneficial during normal training and eating conditions. Also, grip strength was greater in normal condition mice consuming HMB than mice without which suggests that HMB enhances strength gains during normal training conditions. These functional improvements in strength and sensorimotor function may be explained by the greater LBM in normal condition mice consuming HMB.

Interestingly, there seemed to be discrepant results with our DXA data in our catabolic groups. Total body mass of the CH group was significantly greater than that of the C group. Paradoxically, there were less LBM and more FM in the CH group compared to the C group after the 6-week catabolic stress. This finding didn't agree with our gastrocnemius muscle mass and CSA data which were higher in the CH group compared to the C group. Although this wasn't the reason for higher wet weight mass in the gastrocnemius, it might have been due to a higher level of intramuscular fat in the CH group. This notion agrees with the findings of Pedrosa et al. [29] who demonstrated that HMB's parent molecule Leucine; did not reduce body fat in an animal model during the early phase of rapid weight loss. During this catabolic condition, HMB may have been preferentially used as a fuel source instead of fat, thus explaining the higher FM and lower LBM in our catabolic HMB group. This is supported by others who demonstrated that leucine flux, proteolysis, and oxidation are elevated in response to energy deficiency [30,31]. However, it is evident from this study that HMB improves body composition by increasing LBM and decreasing fat mass during normal training and eating conditions.

Protein kinase B (Akt) and mammalian target of rapamycin (mTOR) were analyzed as key markers for protein synthesis [26]. Both catabolic groups expressed significantly higher levels of Akt and mTOR mRNA compared to the ad libitum groups. This finding agrees with Hayashi et al. [32] who also demonstrated an increase in quadriceps Akt levels in calorie restricted (−30%) rats of similar age to the mice used in our study. It seems that the protein synthesis pathway is turned on during a catabolic condition suggesting a different pathway by which Akt is being activated. Akt lies upstream from mTOR. Our catabolic groups upregulated Akt; therefore mTOR was also upregulated [33]. We observed significantly higher levels of Akt mRNA in the gastrocnemius and soleus muscles and significantly higher levels of mTOR mRNA in the soleus muscle in the catabolic group without HMB. These high levels of Akt and mTOR mRNA in the catabolic group without HMB might have been associated with the high mRNA levels of atrogin-1 and MuRF1 and the constant protein turnover occurring in this catabolic condition which was evident in our findings. In addition, our catabolic HMB group attenuated atrogin-1 mRNA and MuRF1 mRNA and also had lower levels of Akt mRNA and mTOR mRNA. The catabolic HMB group seems to have down-regulated this constant protein turnover which seemed to occur in our unique catabolic condition. Our results agree with the findings of Villareal et al. [34] who showed that muscle protein synthesis was not impaired during dietary calorie restriction and weight loss. We postulate that the loss of muscle mass in our study was predominately mediated by the adverse effects of our catabolic condition on muscle proteolysis.

The present study also analyzed E3 ligases, atrogin-1 and MuRF1, to evaluate the role of the ubiquitin–proteasome pathway during our unique catabolic condition. Although we did not observe any differences in atrogin-1 protein expression of the gastrocnemius, elevated mRNA levels of atrogin-1 were shown in the catabolic group mice without HMB. MuRF1 mRNA was elevated in both catabolic groups compared to both normal training groups. MuRF1 mRNA was extremely elevated in the soleus muscle of the catabolic group without HMB. This demonstrates that the ubiquitin–proteasome pathway was up-regulated in our catabolic model and HMB seemed to attenuate this pathway. The increase in atrogin-1 mRNA agrees with Gomes et al. [35], who suggested that atrogin-1 expression is tissue-specific and a critical component of increased proteolysis. One of the most intriguing findings with HMB is its positive influence on attenuating protein degradation, but research has only begun to elucidate this mechanism. Our study is the first to demonstrate that daily HMB intake can attenuate the increase in MuRF1 and atrogin-1 mRNA during prolonged endurance activity combined with calorie restriction. These findings are supported by Smith et al. [5] who showed HMB attenuated the increase in protein degradation in vivo in a MAC16 mouse cancer model. The reduction of atrogin-1 mRNA in our catabolic mice consuming HMB compared to without may be a mechanism for the greater gastrocnemius mass. Therefore, it can be suggested that HMB may blunt muscle wasting in part through suppression of the ubiquitin–proteasome pathway. Based on our mRNA data (Fig. 4), protein synthesis also appeared to be partially blunted; however, we postulate that net protein synthesis may have been greater with HMB during the catabolic condition due to its greater inhibitory effect on the protein degradation pathway (Fig. 5). This may explain why there was greater gastrocnemius mass and CSA in our catabolic HMB group.

In summary, our findings indicate that HMB improves body composition and sensorimotor function during moderate intensity endurance training (fast walk, 3 d/wk) with an ad libitum diet. HMB also attenuates loss of strength, gastrocnemius mass and CSA during catabolic conditions induced by calorie restriction and prolonged endurance activity. The mechanisms underlying the HMB-mediated preservation of muscle loss (i.e. gastrocnemius) under catabolic conditions appear to be through attenuating the ubiquitin–proteasome proteolytic pathway.

The strengths of the present study were that it was well-designed, original and novel to simulate unique catabolic conditions in skeletal muscle, and used extensive in vivo and in vitro measurements. HMB appears to pose anti-catabolic and ergogenic properties during this novel catabolic condition by attenuating loss of muscle and strength. Therefore, HMB has an immediate application to disease and performance of

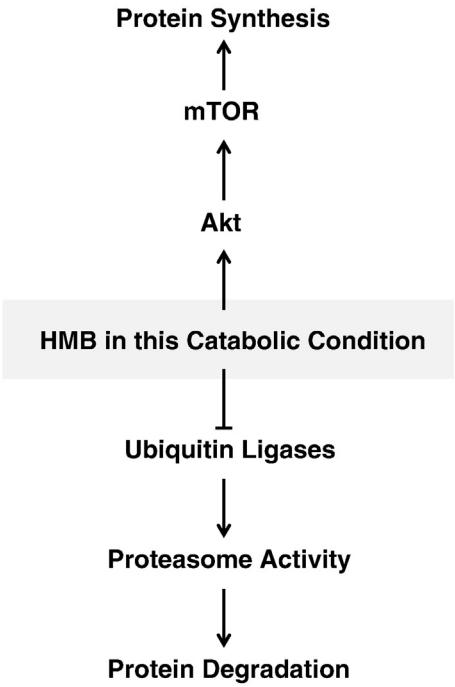

Fig. 5 – Schematic presentation of the potential mechanism of HMB under this catabolic condition.

prolonged tasks by tactical personnel. However, there was no clear explanation for the contradictory findings between lean body mass measured by DXA and gastrocnemius mass and CSA in the catabolic mice consuming HMB. Future studies including clinical trials are warranted to confirm our findings in this animal model.

Author contributions

Bong-Sup Park: Co-first author of study, significant manuscript writer, data acquisition, acquisition of funding.

Paul C. Henning: Co-first author of study, significant manuscript writer, data acquisition, and analysis and interpretation of data.

Samuel C. Grant: Significant manuscript reviewer/reviser, collection of data, and analysis and interpretation.

Won Jun Lee: Acquisition of funding, data acquisition, analysis and interpretation of protein and histology data, and significant manuscript writer.

Sang-Rok Lee: Data acquisition, and analysis and interpretation of data.

Bahram H. Arjmandi: Significant manuscript reviewer/reviser, concept and design, and acquisition of funding.

Jeong-Su Kim: Significant manuscript reviewer/reviser, concept and design, data analysis and interpretation, statistical expertise, and acquisition of funding.

Funding

This work was partially supported by The National Research Foundation of Korea Grant funded by the Korean Government [NRF-2010-356-G00014], The Florida State University and Metabolic Technologies, Inc.

Acknowledgments

This work was partially supported by The National Research Foundation of Korea Grant funded by the Korean Government [NRF-356-2010-1-G00014], The Florida State University and Metabolic Technologies, Inc. The authors acknowledge the efforts Dr. Neema Bakhshalian, Dr. Ihssan S. Masad, Dr. Chris Boehm, Dr. Michael Zourdos, Andy Khamoui and Edward Jo for their technical assistance to this study.

Conflict of interest

The authors declared no conflict to interest.

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