Beta-Hydroxy-beta-methylbutyrate reduces myonuclear apoptosis during recovery from hind limb suspension-induced muscle fiber atrophy in aged rats

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Running head: HMB reduces myonuclear apoptosis during recovery from disuse
ABSTRACT

Beta-hydroxy-beta-methylbutyrate (HMB) is a leucine metabolite that has been shown to reduce protein catabolism in disease states and promote skeletal muscle hypertrophy in response to loading exercise. In this study, we evaluated the efficacy of HMB to reduce muscle wasting and promote muscle recovery following disuse in aged animals. Fisher 344×Brown Norway rats, 34 months of age, were randomly assigned to receive either Ca-HMB (340 mg/kg body weight), or the water vehicle by gavage (n=32/group). The animals received either 14 days of hindlimb suspension (HS, n=8/diet group) or 14 days of unloading followed by 14 days of reloading (R, n=8/diet group). Non-suspended control animals were compared to suspended animals after 14 days of HS (n=8) or after reloading (n=8). HMB treatment prevented the decline in maximal in vivo isometric force output after 2 weeks of recovery from hindlimb unloading. The HMB-treated animals had significantly greater plantaris and soleus fiber cross sectional area as compared to the vehicle-treated animals. HMB decreased the amount of TUNEL-positive nuclei in reloaded plantaris muscles (5.1% vs. 1.6%, p<0.05) and soleus muscles (3.9% vs. 1.8%, p<0.05). Although HMB did not significantly alter Bcl-2 protein abundance, compared to vehicle treatment, HMB decreased Bax protein abundance following reloading, by 40% and 14% (p<0.05) in plantaris and soleus muscles, respectively. Cleaved caspase-3 was reduced by 12% and 9% (p<0.05) in HMB-treated reloaded plantaris and soleus muscles, compared to vehicle-treated animals. HMB reduced cleaved caspase-9 by 14% and 30% (p<0.05) in reloaded plantaris and soleus muscles, respectively, as compared to vehicle-treated animals. Although, HMB was unable to prevent unloading-induced atrophy, it attenuated the decrease in fiber area in fast and slow muscles after hindlimb suspension and reloading. HMB’s ability to protect against muscle loss may be due in part to putative inhibition of myonuclear apoptosis via regulation of mitochondrial-associated caspase signaling.
Introduction

Sarcopenia is the age-associated loss of muscle mass and function (28,29), which is central to the care of geriatric individuals (17). It impairs mobility and increases the susceptibility to muscle injury (30), thereby leading to a decrease in the independence and the quality of life in the elderly (15), as well as to increase the risk for serious falls (85,86). In addition, sarcopenia exacerbates the loss of muscle function and the quality of life in aged individuals with restricted mobility or extended hospitalization. While sarcopenia is multifactorial, apoptosis has been shown to play a role in the events that lead to muscle loss with aging.

Several laboratories have shown that apoptosis is a significant contributor to muscle degeneration and sarcopenia (1-3,22,45,49,50,57,70,72,76,80,84). However, apoptosis in skeletal muscle is uniquely different than cell death that has been described classically in other tissues and cells. Most importantly, skeletal muscle is multi-nucleated, so that the removal of one myonucleus by apoptosis will not produce widespread or extensive muscle cell death. However, elimination of a myonucleus will lead to loss of gene expression within the cytoplasmic domain that had been controlled by that myonucleus. This loss of gene control cannot be fully assumed by an adjacent myonucleus, and after some threshold of nuclear loss, the muscle fiber will undergo atrophy. Evidence that not all myonuclei in a single myofiber become apoptotic during muscle loss has been observed in experimental denervation and denervation-associated diseases (31,68).

Three primary apoptotic pathways have been implicated in apoptotic signaling of skeletal muscle fibers. These include mitochondria-dependent (intrinsic), death receptor-mediated (extrinsic), and sarcoplasmic/endoplasmic reticulum-calcium stress-induced pathways (8). Several lines of data suggest that the intrinsic mitochondrial associated pathway has an important role in apoptotic signaling in muscle under conditions of aging (16,22) and under cardiovascular pathologies (59,60). Furthermore, apoptotic signaling increases in aged muscles
during periods of disuse (9,23,46,49,73,76). However, it is not known to what extent apoptotic
signaling is altered during muscle reloading in aged animals after disuse.

Aging reduces the ability of muscle to recover after immobilization or disuse in humans
(38,83) and animals (39,94). Furthermore, muscle growth is suppressed with aging, because
the extent of hypertrophy is attenuated in muscles from aged animals as compared to young
adult animals (5,20,21,69) and this reduced muscle growth with aging is associated with
increased apoptotic signaling (7,8,71). As reloading after immobilization has been shown to
reduce mitochondrial-associated apoptotic signaling in skeletal muscle (88) of young adult
animals, and younger animals have better recovery following disuse than old animals (39,94),
we were interested in identifying approaches that would reduce apoptotic signaling and thereby
improve muscle structure and function during recovery following disuse in old animals.

One potential candidate for improving muscle mass and growth under various conditions
is β-hydroxy-β-methylbutyrate (HMB). HMB, a metabolite of the essential branched-chain amino
acid leucine, has been found to reduce muscle wasting associated with trauma and cancer
cachexia (11,14,55,87). Furthermore, HMB supplementation has been reported to improve
muscle function in the mdx mouse model for Duchenne muscular dystrophy, which, has a high
degree of muscle degeneration and regeneration (55). Furthermore, HMB has been shown to
attenuate fiber atrophy and damage during limb immobilization of adult mice (82).

HMB has been reported to reduce muscle atrophy and increase muscle hypertrophy by
inhibiting muscle degradation (77), in part, from greater Akt phosphorylation (42) and improved
anabolic signaling via the m-TOR pathway (11). However, although less well studied, HMB has
also been shown to reduce apoptosis in human myoblasts under conditions of serum-
starvation or staurosporine-induced apoptosis (42). We have recently found that serum
starvation induces myoblast apoptosis that was accompanied by an increased mitochondrial
release of cytochrome c, cleaved caspase-9 and apoptosis inducing factors as a result of
increased mitochondrial associated apoptotic signaling (Yan Wang, unpublished observations).
Taken together, the findings from Kornasio and colleagues (42) and our lab indicate that HMB might be a potential candidate that will protect against mitochondrial-associated apoptotic signaling.

It is well established that muscle atrophy induced by hindlimb suspension in rodents is associated with mitochondrial-associated apoptotic signaling (9,23,46,49,73), and reloading after immobilization has been shown to reduce mitochondrial-associated apoptotic signaling in skeletal muscle (88). Together, these observations lead us to ask if HMB might also protect against mitochondrial-associated apoptotic signaling after hindlimb suspension and subsequent reloading of skeletal muscle in rats. In this study, we tested the hypothesis that HMB would reduce myonuclear apoptosis in the hindlimb muscles of aged rats in response to disuse and reloading following disuse. Our data show that HMB attenuated the decrease in muscle fiber area in both fast and slow muscles after hindlimb suspension. Furthermore HMB reduced muscle atrophy and improved muscle force during reloading after disuse, and also reduced myonuclear apoptosis and abundance of pro-apoptotic proteins Bax and caspase-3 during recovery of hindlimb muscles of aged rats after disuse.

Materials and methods

Animal care. Sixty-four male Fisher 344×Brown Norway rats, 34 months of age, were obtained from the National Institute on Aging (NIA) colony that was housed at Harlan (Indianapolis). The animal care standards were followed by adhering to the recommendations for the care of laboratory animals as advocated by the American Association for Accreditation of Laboratory Animal Care and following the policies and procedures detailed in the Guide for the Care and Use of Laboratory Animals as published by the U.S. Department of Health and Human Services and proclaimed in the Animal Welfare Act (PL89-544, PL91-979, and PL94-279). All experimental procedures carried approval from the Institutional Animal Care and Use Committee from the West Virginia University.
Hindlimb suspension (HS) and reloading (R) after HS. The rats were randomly assigned to ambulatory control (C, n=32), hindlimb suspended (HS, n=16) or reloaded (R, n=16) groups. Hind limb suspension was conducted for 14 days as described previously (58). Briefly, orthopedic tape was applied along the proximal one-third of the tail then placed through a wire harness that was attached to a fishlike swivel at the top of a specially designed hindlimb suspension cage. This provided the rats with 360° of movement around the cage. Sterile gauze was wrapped around the orthopedic tape and was subsequently covered with a thermoplastic material (Vet-Lite; Veterinary Specialty Products, Boca Raton, FL). The exposed tip of the tail was monitored to ensure that it remained pink, indicating that HS did not interfere with blood flow to the tail. The suspension height was monitored daily and adjusted, to prevent contact between the hindlimb and any supportive surface in the cage. The suspension angle did not exceed 30°. The forelimbs maintained contact with a grid floor, which allowed the animals to move, groom themselves, and obtain food and water freely. In the R group, the rats were released from HS and allowed normal cage ambulation for 14 days. In the control group, the rats maintained normal mobility, and they moved freely around their cages.

Nutritional treatment with HMB. Previous studies have reported reduced muscle wasting in rodents in response to cancer cachexia with HMB supplemented at 250 mg/kg body weight (78). This is approximately six times the dose of most studies in humans (62). Our pilot experiments indicated that low doses of HMB were not an effective deterrent for suppressing muscle loss or apoptotic signaling in muscles of aged rats during hindlimb suspension, whereas a dose of 340 mg/kg that was ~8 times that of human dosing was effective. Therefore, 8 conscience animals in each experimental group received 340 mg/kg of Ca-HMB (TSI Health Sciences) dissolved in distilled water (170mg/ml), or 1ml of the vehicle (distilled water) by gavage feeding. Animals in the HS and R groups were pretreated with Ca-HMB or the vehicle for 1 week prior to HS. This
allowed the animals to readily accommodate to gavage when they were tail suspended. The
animals were given free access to a diet of Purina 5008 rat chow (Ralston Purina, St. Louis,
MO) and water over the course of the study.

Research design. Muscle data were obtained from animals treated with HMB or the vehicle.
Two groups of ambulatory non-suspended control animals were used. Sixteen cage control
animals received the vehicle (CC-Veh) and 16 animals were cage controls that received HMB
(CC-HMB). The cage control animals that were examined 14 days after the initiation of the study
were controls for the HS groups (HS-Con; n=8). A second cage control group of 8 animals/diet
group was examined 28 days after the initiation of the study (R-Con; n=8). These animals were
used as controls for the animals that received HS for 14 days followed by 14 days of reloading.
8 animals/diet group were examined after 14 d of HS and another 8 animals/diet group were
examined after 14d of HS followed by 14 d of recovery.

Force measurements. All force measurements were made while the animals were
anesthetized with 98% oxygen and 2% isoflurane gas (53,65). The animals were placed supine
on the heated X–Y positioning table using a custom built rat dynamometer (19), with the left foot
was secured to the footplate at an ankle angle of 90°. Vertical forces were translated to a load
cell transducer in the load cell fixture on the footplate. Platinum stimulating electrodes (Grass
Medical Instruments, Quincy, MA) were inserted subcutaneously to span the tibial nerve in the
popliteal fossa. The maximal isometric force of the plantar flexor muscle group was evaluated
by stimulating the tibial nerve using supramaximal square wave pulses that were 4 V, 100Hz for
3 s, using a SD9 stimulator (Grass Medical Instruments, Quincy, MA). Maximal force was
determined using Labview based software. The maximal forces for three isometric contractions
were averaged for each data point. Maximal isometric force measurements were made before
14 days of HS (day 0), after 14 d of HS, and 14 days after-reloading the hind limbs following the 14 day HS period.

**Body weight and tissue preparation.** Each animal was weighed at the beginning of the experiment, following 14 days of HS, and after 14 days of reloading. At the end of the experimental period, and with the animals deeply anesthetized, the soleus and the plantaris muscles were removed from both limbs, then blotted, and weighed. Following this procedure, the animals were euthanized by removing the heart. A block obtained from the mid-belly of the muscle was embedded in optimal cutting temperature (OCT) compound (Tissue-Tek; Andwin Scientific, Addison, IL), snap frozen in liquid nitrogen cooled isopentane, and stored at -80°C. The remainder of the muscle was snap frozen in liquid nitrogen and stored at -80°C until needed for subsequent analyses.

**Identification of apoptotic nuclei.** 10-µm-thick frozen cross sections from soleus and plantaris muscles were mounted on charged microscope slides (Fisher Scientific, Pittsburgh, PA). Apoptotic nuclei were identified by labeling the sections with fluorescent labeling of terminal dUTP nick-end labeling (TUNEL) (11684795910; Roche Applied Science, Indianapolis, IN) and lamina, using methods that were modified slightly from that which was previously reported for our lab (75). Briefly, the tissue sections were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) and permeabilized with 0.1% Triton X-100 in PBS. The tissue was then incubated at 4°C over night with a rat anti-lamina monoclonal antibody (MAB 1914, Millipore, Billerica, MA) to visualize the basal lamina of each muscle fiber. The sections were then incubated with donkey anti-rat rhodamine conjugated second antibody (712-025-150, Jackson ImmunoResearch Laboratories, West Grove, PA) along with the TUNEL reaction mixture in a humidified chamber at 37°C for 1 h in the dark. Omission of the TdT enzyme in the TUNEL
reaction mixture on one the tissue sections on each slide was included as a negative control. The sections were mounted with 4',6-diamidino-2-phenylindole (DAPI) to visualize all nuclei (Vectashield mounting medium; Vector Laboratories, Burlingame, CA and viewed under a Zeiss LSM 510 Meta confocal microscope (Carl Zeiss Microimaging Inc. Thornwood, NY). The number of TUNEL- and DAPI-positive nuclei that were immediately adjacent to, or beneath the basal lamina were counted. Data were expressed as an apoptotic index, which was calculated by counting the number of TUNEL-positive nuclei divided by the total number of nuclei (i.e., DAPI-positive nuclei). The apoptotic index was determined from ~1200 fibers, which were obtained from four non-overlapping regions of each tissue cross section.

**Fiber morphology.** Muscle fiber cross sectional area (CSA) was determined by planimetry from 750-1200 fibers that were obtained from four non-overlapping regions of each tissue cross section stained for lamina. Fiber CSA was calculated by ImageJ software (NIH). Muscle fiber CSA was not subdivided by fiber type. However, the plantaris muscle in Fisher Brown Norway rats are composed primarily of type II fibers (32), whereas the soleus is composed of ~ 90% type I fibers (32,81). Thus the sampling of fiber CSA likely represented primarily type II fibers in the plantaris muscle and type I fibers in the soleus muscle.

**Western immunoblots.** Approximately seventy-five micrograms of muscle was homogenized in ice-cold RIPA buffer (1% Triton x-100, 150 mM NaCl, 5 mM EDTA, 10 mM Tris; pH 7.4), containing protease inhibitors (P8340, Sigma-Aldrich, St. Louis, MO), and phosphatase inhibitors (P2850; P5726, Sigma-Aldrich). The muscle homogenates were centrifuged at 1000 x g for 5 minutes at 4°C, and the protein content of the supernatant was measured (500-0116; BioRad, Hercules, CA). Forty micrograms of protein were loaded into each well of a 4-12% gradient polyacrylamide gel (NP0335BOX; Invitrogen, Carlsbad, CA) and separated by routine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for 1 hour at 120V. The
proteins were transferred to a nitrocellulose membrane for 1.5 hours at 25V. Non-specific protein binding was blocked by incubating the membranes in 5% nonfat milk in Tris-buffered saline containing 0.05% Tween 20 (TBST) at room temperature. The membranes were incubated (1:1000) overnight at 4°C, with primary antibodies directed against Bcl-2 (#2876, Cell Signaling Technology, Boston, MA), Bax (#2772, Cell Signaling), cleaved caspase-3 (#9664, Cell Signaling) and cleaved caspase-9 (#9509, Cell Signaling). The membranes were washed in TBST and incubated in appropriate dilutions of secondary antibodies (diluted in 5% non-fat milk) conjugated to horseradish peroxidase (Sigma-Aldrich, St. Louis, MO). The signals were developed using a chemiluminescent substrate (Lumigen TMA-6; Lumigen, Southfield, MI) and visualized by exposing the membranes to x-ray films (BioMax MS-1; Eastman Kodak). Digital records were captured with a Kodak 290 camera, and protein bands were quantified using 1D analysis software. The bands were quantified as optical density X band area and expressed in arbitrary units.

Statistical analysis. The results are reported as means ± SD. Differences in means between groups were determined by a Two-Way (2x2) Analysis of Variance with the factors of diet (HMB vs. vehicle) and treatment (HS, cage control). A repeated measures ANOVA was used for longitudinal data analyses of the animals in the recovery group, for assessments before HS, after HS and in recovery. Bonferroni post hoc analyses were performed between significant means. Chi-squared analyses were conducted between experimental groups for the fiber area-frequency data. Statistical significance was set a $p<0.05$. 
Results

**Body weight.** The bodyweight of the animals did not differ among the experimental groups at the beginning of the study (Supplemental Table S1). The body weight of the vehicle-treated cage control and HMB cage control animals did not change over the course of the study. Figure 1 presents longitudinal data for animals in the recovery group and their respective control groups. By contrast, 14 days of HS significantly lowered the animals’ body weight by ~15% (p<0.05) after treatment in both HMB and water treated groups (n=16/group) but there was no difference between treatment groups. Longitudinal assessments of the bodyweight did not differ between HS and the reloading period of the animals that were in both treatment groups (n=8 per group) (Figure 1).

**Maximal isometric force.** HMB attenuated the loss of force during recovery after unloading, but it did not prevent the decline in force production after HS. Figure 2 shows the longitudinal data for animals in the recovery group compared to the cage control animals for the two diet groups. Maximal isometric force was not different among the groups prior to HS. For animals in the R group, 14 days of HS reduced maximal in vivo plantarflexor isometric force by 34.3% in vehicle-treated animals, and by 23.7% in HMB-treated animals (n=16 per group), however, this did not represent a statistically significance difference between HMB and control groups. In contrast, there was a significantly greater loss in maximal isometric plantarflexor force of the vehicle-treated animals (42.4%) than HMB-treated animals (27.3%) in the R group after reloading (n=8 per group) (P<0.01, Figure 2) as compared to the respective cage control groups. The force data for animals that were examined and euthanized after 14 days of HS, did not differ from animals in the R group that were examined after 14 days of HS (Supplemental Table S2).
**Muscle wet weight.** HMB did not reduce the extent of HS-induced atrophy, but it did improve muscle wet weight in the plantaris muscles of the recover group that were reloaded after HS, relative to the vehicle control animals. HS induced a significant decrease in the wet weight of the plantaris (19%) and soleus (15%) muscles (P<0.001), of both HMB and vehicle-treated animals (n=16 per group). Gastrocnemius muscle wet weight was not significantly reduced by HS and did not change during recovery in either diet group (Supplemental Table S3). There was no significant difference in the extent of plantaris or soleus muscle mass loss between the experimental groups after 14 days of HS. Reloading prevented any further decline in soleus and plantaris muscle wet weight, but it did not fully reverse the muscle loss induced by HS. HMB treatment significantly improved plantaris muscle weight after 14 days of reloading relative to vehicle-treated animals (n=8 per group) (Figure 3A). However, HMB did not provide any protective effect against HS-induced loss in the soleus muscle wet weight, nor did it improve soleus muscle wet weight recovery after 14 days of reloading, relative to the vehicle-treated animals (Figure 3B).

**Changes of muscle fiber CSA.** HMB appeared to reduce the extent of fiber atrophy in both the slow soleus and the fast plantaris muscles that occurred after HS or reloading. HS dramatically decreased mean fiber CSA in both plantaris (Figure 4A) and soleus (Figure 4B) hindlimb muscles. However, the HS-induced decrease in fiber CSA of the vehicle-treated animals was greater than in the HMB-treated animals for plantaris (48.8% vs. 26.4%, p <0.05) and soleus muscles (45.6% vs. 32.5%, p <0.05). HMB did not further improve fiber CSA in either plantaris (Figure 4A) or soleus (Figure 4B) muscles after 14 d of reloading as compared to HS. The fiber area-fiber frequency distribution for the plantaris (Figure 4C) and soleus (Figure 4D) are shown for the recovery group. After 14 days of recovery, the muscle fibers in both the plantaris and soleus were still shifted to the left in the fiber area-frequency distribution, relative to the distribution of the control muscles. Chi-squared analyses showed that the frequency of fibers <
1500 µm² was significantly greater in the plantaris and soleus muscles of vehicle-treated animals as compared to HMB-treated animals that had recovered for 14 days following HS. The distribution of the fiber area-frequency distribution after HS (Figure 4C, Figure 4D) was similar to that shown for recovery (data not shown).

**Apoptotic myonuclei as identified by TUNEL labeling.** The apoptotic index as indicated by the number of TUNEL positive myonuclei to total myonuclei, was significantly increased by HS, and reloading following HS, relative to muscles from cage control animals, but HMB attenuated the apoptotic index in both HS and recovery muscles. HS significantly increased the number of TUNEL positive nuclei in both plantaris (Figure 5A) and soleus (Figure 6A) muscles. Although there were some regional differences within tissue sections, TUNEL-positive nuclei occurred throughout each tissue cross section that was obtained from plantaris and soleus muscles following HS. The apoptotic index was significantly increased in plantaris (9.9 fold, \( p<0.05 \)) and soleus (3.2 fold \( p <0.05 \)) muscles of vehicle-treated animals as compared to ambulatory control animals. Although HMB treatment suppressed myonuclear apoptosis, it did not eliminate it. HS increased the apoptotic index in both plantaris (3.0 fold, \( p<0.05 \)) and soleus (1.8 fold, \( p<0.05 \)) muscles of HMB-treated animals compared to ambulatory control animals. The apoptotic index was significantly greater (\( p<0.05 \)) in both plantaris (Figure 5C) and soleus muscles from vehicle as compared to HMB-treated animals (Figure 6C). Apoptosis, as identified by TUNEL labeling, remained high during reloading following HS, especially in vehicle-treated muscles (Figure 5B, 6B). HMB significantly suppressed TUNEL labeling in the myonuclei of reloaded plantaris (Figure 5C) and soleus (Figure 6C) muscles relative to vehicle treated animals. The ratio of TUNEL positive myonuclei to total myonuclei in reloaded, HMB treated soleus was not different from the ratio of TUNEL positive to total myonuclei in soleus cross sections from in cage control animals (Figure 6C).
**Apoptotic signaling proteins.** HMB treatment suppressed the HS-induced increase in the pro-apoptotic proteins after HS and reloading, as compared to vehicle-treated animals. Pro-apoptotic proteins associated with mitochondrial apoptotic signaling significantly increased in abundance in hindlimb muscles after HS, and remained elevated during reloading. Pro-apoptotic proteins including Bax (Figure 7), cleaved caspase-9 (Figure 8), and cleaved caspase-3 (Figure 9) all significantly increased ($p<0.05$) after both HS and reloading. HMB significantly suppressed the protein abundance for Bax (Figure 7), cleaved caspase-9 (Figure 8), and cleaved caspase-3 (Figure 9) in plantaris and soleus muscles after both HS and reloading conditions ($p<0.05$). The abundance of pro-apoptotic signaling proteins was similar in the plantaris (Figure 7A, 8A, 9A) and soleus (Figure 7B, 8B, 9B) muscles after HS and reloading, and this was not altered by HMB. It was interesting to note that HMB significantly reduced the protein abundance of Bax (Figure 7B), and cleaved caspase-3 (Figure 9B) in control muscles of ambulatory cage control animals (HS Con and R Con). Both HS and reloading after HS significantly increased the anti-apoptotic protein, Bcl-2, in plantaris (Figure 10A) and soleus (Figure 10B) muscles by ~100% ($p<0.05$), but there was no significant difference between HMB and vehicle-treated muscles after either HS or reloading after 14 days of HS.

**Discussion**

HMB supplementation has been proposed to prevent muscle damage (41), lower protein degradation (77), and directly stimulate skeletal muscle protein synthesis (91). In addition, HMB has been shown to reduce the extent of apoptosis in muscle cells *in vitro* (42), although the potential for HMB to moderate apoptosis in skeletal muscle *in vivo* has not been previously studied. In this study, we provide the first evidence for a positive influence of HMB on the reduction of pro-apoptotic signaling in response to 14 days of hindlimb-induced muscle disuse and also in response to 14 days of muscle reloading following hindlimb suspension in aged rats.
Although HMB could not fully prevent HS-induced body weight or muscle loss in response to unloading, it: (i) prevented further force loss during reloading after unloading, (ii) improved muscle mass in the plantaris muscles that were reloaded after HS, (iii) blunted the extent of fiber atrophy in both fast and slow skeletal muscles in response to unloading and reloading after disuse, (iv) significantly attenuated myonuclear apoptosis induced by HS, (v) decreased the apoptotic index after reloading following HS in both plantaris and soleus muscles; and (vi) reduced mitochondrial apoptotic signaling as indicated by lower levels of cleaved caspase-3, cleaved caspase-9 and Bax protein abundance in reloaded plantaris and soleus muscles.

**HMB protects loss of muscle function.** Studies from our lab and others (6,58,74-76,91) have demonstrated that HS dramatically reduces skeletal muscle mass and strength in both young and aged rats. In contrast to young adult animals which recover plantaris and soleus muscle weight within 14 days of reloading after 14 days of HS (Figure S1), the data in the current study (Figure 3) show that there is no recovery of muscle mass of previously unloaded muscles after 14 days of HS followed by 14 days of reloading (recovery) in the soleus of aged Fisher Brown Norway rats, although there is some improvement in the plantaris weight and muscle weight/bodyweight of HMB-treated aged rats after 14 days of reloading (Figure 3). The failure to recover muscle lost during HS after reloading of the hindlimbs in aged rats may be explained in part, by elevated pro-apoptotic signaling proteins in these muscles after both HS and reloading following HS. However, in young rats, full recovery of muscle mass during reloading after HS is accompanied by a reduction in pro-apoptotic signaling (Figure S2; S3: S4). HMB treatment did not improve this recovery in young animals, but it did increase (but not fully) muscle mass in the plantaris of aged rats during the 14 day recovery period following HS. While the extent of recovery of reloaded muscles following HS was similar in HMB and vehicle treated animals, we could not determine from this study, if the recovery of muscle mass following HS in
young animals occurred more rapidly in the HMB fed group as compared to the vehicle treated group.

As the gastrocnemius muscle is the greatest contributor to plantar flexion force, yet HS and reloading after HS did not significantly alter gastrocnemius muscle mass (Supplemental Table S3), changes in plantar flexion isometric force after HS and also in for animals in the R group, must be primarily the result of changes in function of the soleus and plantaris muscles. Our data are consistent with observations from young adult rats (90) and mice (34), which show that reloading after disuse did not improve the production of muscle force. Nevertheless, HMB helped to prevent any further reduction in maximal isometric force in the plantar flexor muscles following reloading after HS as compared to the vehicle-treated animals (Figure 2). The age-associated increase in the potential for muscle injury during reloading (12), and/or the widespread diminished ability of muscles from aged animals to respond positively to loading paradigms (5,18), likely also contribute to the lack of functional improvement with reloading. We did not complete a full time response curve of the responses for either HS or recovery following HS. Therefore, it is not possible to know if the muscle mass was lost in both vehicle and HMB groups during the early parts of reloading and then was restored more fully or rapidly during the subsequent days of recovery in the HMB group vs. the control group, or, if HMB provided a protection against the loss of muscle mass and muscle function during reloading. Either scenario would be consistent with the modest protection that HMB provides against loading-induced muscle damage and repair (92).

HMB did not alter the body weight of young adult animals during HS or during the recovery period after HS (Supplementary Table S4). Furthermore, HMB did not improve the recovery of muscle mass in young animals after hindlimb unloading, because muscle restoration was complete by 14 days following HS in young adult rats (Supplementary Table S5; Supplementary Figure S1). In contrast, HMB reduced the extent of recovery-induced atrophy during reloading after HS in the plantaris muscle of aged animals (Figure 3A). This was a
muscle specific effect, because soleus muscle wet weight was not different in the HMB and vehicle groups during recovery, whereas plantaris muscle wet weight was greater in the HMB vs. the vehicle treated group. Nevertheless, fiber CSA was greater in the HMB group in both the plantaris and soleus muscles during recovery.

It was interesting to find a lack of preservation of muscle wet weight after HS (Figure 3) in the soleus or plantaris, despite preservation of muscle fiber CSA. We had expected that any changes in muscle wet weight would be reflected by similar changes in muscle force production. However, isometric force seemed to track muscle fiber CSA more closely than muscle wet weight. For example, in aged animals, isometric plantarflexor force declined with HS, as did plantaris and soleus fiber CSA, yet force was greater in HMB treated muscles after reloading, and fiber CSA was also greater in both plantaris and soleus muscles from HMB treated animals as compared to control animals. Although speculative, several possibilities could explain this. First, HS may have reduced total plantaris and soleus fiber number in the muscles of aged animals (perhaps via apoptosis), which in turn, may have contributed to overall lower muscle volume and mass (35), and this was not prevented by HMB. If this occurred then we would not expect that muscles in old animals could recover without adding back the lost fibers (which might be difficult or impossible), and therefore fiber hypertrophy would be the primary mechanism leading to improved muscle mass and function during reloading. Some compensation of increasing fiber size by HMB during HS might buffer the loss of force, if the cross bridges were all capable of generating maximal force (i.e., moving from the weakly bound to strongly bound positions (47). This may have been the case (Figure 2) although this was not statistically significant. Secondly, if some fibers fused together during remodeling associated with HS or reloading following HS, there could be larger fiber sizes without any change in muscle mass. Likewise, with reloading, new fiber accumulation (33) or fiber splitting could have occurred as potentially the result of incomplete fiber repair as shown in human muscles after extreme loading conditions (27) and in disease (61). Further, fiber hypertrophy in split or new
fibers could then have contributed to increased muscle mass and force in the plantaris during
reloading after disuse. In contrast, the larger fiber CSA in the soleus would not be expected to
contribute to any improvement in muscle mass or force if this only reflected an increased in the
number of fused fibers. Another possibility is that activated satellite cells fused to existing
muscle fibers and began protein synthesis during the atrophic process, in an attempt to offset
muscle wasting, but this would result in minimal changes in muscle wet weight until sufficient
time had occurred to accumulated new protein. In addition, the activated satellite cells during
HS would at least initially express immature myosin isoforms (10,93), which would not have
contributed to marked improvement in muscle force, whereas during recovery, there would have
been sufficient time for the myosin isoforms in the plantaris to mature and become expressed,
fused to the existing fibers and fully integrated in sarcomeres, so that they could both enlarge
fiber CSA and contribute to the production of force. It is possible that maturation of the soleus
muscle isoforms was incomplete and this would reduce the contribution to improving overall
muscle function. It was however, interesting that the loss of muscle force production tracked the
changes in fiber cross sectional area better than muscle wet weight. This observation may have
important implications because it suggests that fiber CSA may be a better predictor of muscle
function (isometric force) than muscle wet weight. We did not identify cross sectional area for
individual fiber types, but rather provided a random sampling across the predominately type II
myosin containing plantaris muscle and the type I myosin containing soleus muscle. It is
possible that one fiber type might be a better indicator of changes in muscle function, but we
cannot determine this from the current data. As the gastrocnemius muscle did not atrophy
during HS or hypertrophy during reloading following HS (Supplemental Table S1), the changes
in force were presumably the result of remodeling of the plantaris and soleus muscles, but since
the plantaris muscle is larger, it would make the greatest contribution to the changes in
isometric muscle force in the hindlimb of the aged rats.
Apoptosis with muscle disuse and aging. The incidence of apoptosis (Supplemental Figure S2) and pro-apoptotic signaling (Supplemental Figures S3, S4), are very low in muscles from young animals and although apoptosis increases with hindlimb suspension, it is still lower than that observed for muscles of aged animals. Apoptosis has been suggested to play an important role in the development of aging-associated sarcopenia and apoptotic signaling is elevated during periods of muscle disuse (4,23,46,54). The results the current study are consistent with previous data that have shown that muscle atrophy induced by unloading, increases apoptotic signaling in both the plantaris and the soleus muscles of aged Fisher 344×Brown Norway rats (54,70,76). The inability to improve muscle mass or strength in response to reloading, may be the result of an aging-associated decline in myonuclei (in part as a result of elevated myonuclear apoptosis), and therefore, a lower transcriptional capability in muscles of aged animals (52). This speculation is supported by our observations in the current study that apoptosis is elevated in hindlimb muscles of aged rats during conditions of both unloading and reloading. In the present study, HS significantly increased the number of TUNEL positive nuclei to total nuclei in cells from both plantaris and soleus muscles of aged animals, and this was accompanied by an increase in the pro-apoptotic proteins Bax, caspase-9, and caspase-3 (Figures 7-9), which together are involved in the mitochondria-dependent apoptotic signaling pathway (9). Interestingly, the anti-apoptotic protein content of Bcl-2 was also increased after HS and reloading (Figure 10). We cannot rule out the possibility that apoptotic signaling may have occurred by a combination of muscle and non-muscle cells (e.g., endothelial cells) which reside inside muscles (i.e., within the basal lamina) in vivo. Furthermore, the apoptotic index could overestimate the number of nuclei that would be lost to apoptosis, if DNA damage was eventually repaired in some of the TUNEL positive nuclei. However, the immunocytochemical data clearly showed that the TUNEL positive nuclei that were quantified were of myonuclear or satellite cell origins, and not non-muscle nuclei (Figure 5-6). Furthermore, we anticipate that many of the TUNEL positive nuclei would result in loss of nuclei in unloaded and reloaded
muscles as a result of apoptosis, and this would reduce the ability to maintain transcriptional
regulation of muscle proteins, and thereby, potentially contributing to fiber atrophy. Apoptosis
was much less severe in muscles of young adult animals (Figure S2) as compared to muscles
in aged animals (Figure 5, 6) and this was consistent with lower deficits in function in young
animals (Table S5) as compared to aged animals that were subjected to the same conditions
(Figure 2; Table S2). Together these findings suggest that apoptosis contributes to muscle loss
of aged hosts during stresses (either unloading or reloading after disuse) but it plays little
importance in remodeling of muscle of young hosts that are subjected to similar stresses.

The data in this study suggests that there is not a linear relationship between increased
signaling for nuclear apoptosis and muscle mass. This is because the outcome of apoptosis in
multi-nucleated skeletal muscle differs from apoptosis in the classical one-cell systems (8,9). In
skeletal muscle nuclei can be eliminated without a substantial change in muscle mass or loss of
muscle fibers. After some critical threshold (and we do not know what point that exactly occurs)
fiber atrophy would need to occur, as the existing nuclei would presumably be unable to
maintain the given fiber size (8,9). After fiber CSA has been decreased as a result of elimination
of enough nuclei, there would presumably be some measurable loss of muscle wet weight.
However, we would anticipate that the changes in fiber cross sectional area would precede and
be more sensitive to apoptotic changes in muscle than would muscle wet weight. Thus, changes
in fiber CSA should be better predictors of apoptotic changes in muscle as compared to muscle
wet weight, and the data in this study supports this possibility. A second factor which offsets the
loss of muscle mass that would have occurred as a result of an increase in pro-apoptotic
signaling is the observed increase in anti-apoptotic proteins such as Bcl-2 (Figure 10;
Supplemental Figures S3, S4).

In this study, we show that even during muscle reloading following 14 days of unloading,
there was apoptosis ongoing in both plantaris and soleus muscles albeit less than that occurring
during HS alone. Thus, the common stimulus triggering apoptosis in skeletal muscles of old
animals is unlikely to be solely due to the presence or absence of contractile tension in muscles, or tension that is sensed by the extracellular matrix. One potential candidate for triggering apoptosis under both unloading and loaded conditions, is that the high level of oxidative stress that exists in both unloaded (44,74) and acutely loaded (65-67) muscles of aged rats. Another possibility is a systemic elevation of cytokines in response to loading or unloading (56,58).

**HMB regulation of apoptosis in unloading and reloading.** HMB supplementation has been previously shown to blunt muscle loss in critically ill patients (43) including patients with inflammatory diseases (37,48,51). This is due, at least in part, to reductions in proteasome activity (36). In addition, there is evidence that HMB has potential to improve strength gains in response to resistance exercise in 70-year old males and females (89). Our data build upon these observations by showing that HMB can reduce unloading-reloading muscle fiber area losses in fast skeletal muscle, and this is associated with reduced apoptotic signaling in aged fast (i.e., plantaris) skeletal muscles. For example, HMB significantly attenuated the increase of the pro-apoptotic proteins (e.g. Bax, cleaved caspase-9, cleaved caspase-3) and the number of apoptotic nuclei in both plantaris and soleus muscles after HS and reloading in aged rats although the positive effects of HMB were less dramatic in the soleus muscle. Thus, the effect of HMB appeared to suppress apoptotic signaling to a greater extent in the predominantly fast myosin containing plantaris muscle compared to the predominately slow myosin containing soleus muscle. The data from this study do not provide an explanation for this finding. While speculative, it is possible that the plantaris muscle is more sensitive to HMB, perhaps the degree of apoptosis was more severe in the plantaris as compared to the soleus muscle (apoptotic index, 9.9 fold vs. 3.2 fold).

Although beyond the scope of this study, additional studies are required to determine if the levels of oxidative stress or cytokines are similar in the fast plantaris and slow soleus muscles of aged animals under conditions of unloading or reloading, and if HMB affects these
potential triggers for apoptosis. This is a possible mechanism worth examining because HMB is known to down regulate lipopolysaccharide induced oxidative stress (64) as well as the cytokine tumor necrosis factor-alpha (TNF-α-induced) (25,26) NFkB (63) activation in cachectic muscle. Oxidative stress would be expected to be greater in the plantaris than the soleus, because slow muscles like the soleus have a greater antioxidant capacity (40) (e.g., greater mitochondria localized manganese superoxide dismutase, glutathione etc.), than fast muscles like the plantaris. In this scenario, the soleus would have a lower need for buffering oxidative stress, and therefore HMB would have a lower impact in the soleus than in the plantaris muscle. Another possibility is that the mechanisms that regulate muscle loss are different in the fast plantaris and slow soleus muscles. For example, the frequency of TUNEL positive myonuclei appeared to be greater in the plantaris vs. the soleus muscle after HS and R conditions. It is therefore possible that multiple apoptotic pathways including cytokine and mitochondrial pathways were activated in the atrophied plantaris muscle (58,73), whereas primarily mitochondrial associated signaling was more prevalent in the soleus muscle during HS and R (46). Although speculative, it is feasible that HMB may have been more effective at suppressing apoptotic pathways associated with muscle loss in the plantaris as compared to pathways regulating muscle loss in the soleus of aged rats.

The apoptotic index was not altered by HMB in control muscles. However, it appears that part of the basal apoptotic signaling pathway was suppressed by HMB treatment. This can be seen by significantly lower abundances of cleaved capase-9 and cleaved caspase-3 in plantaris and soleus muscles of control animals, which had been supplemented for 5 weeks (R Con animals). In some cases, the shorter supplemental period of 3 weeks (HS Con animals) failed to have lower caspase signaling in the plantaris muscle as compared to the vehicle ambulatory control animals. Presumably, resetting the basal levels of pro-apoptotic signaling protein abundance by HMB (as seen in the control animals) should protect the subsequent apoptotic stresses that would be introduced to the skeletal muscles. Thus, some of the benefit
of HMB suppressed apoptotic signaling in HS and reloading conditions, may be a resetting of
the apoptotic threshold.

Mitochondrial apoptotic signaling proteins are altered by HMB treatment, and therefore it
is likely that mitochondrial structure and/or functions improved by HMB during severe stresses
such as reloading after HS. This is possible since HMB stabilizes sarcolemma HMG-CoA
reductase (reviewed in (91)). Mevalonic acid, produced from HMG-CoA reductase is a
precursor of coenzyme Q and dolichols, which play a major role in mitochondrial electron
transport function and myocyte proliferation. Additional studies are needed to determine if HMB
affects apoptosis by stabilizing the function of mitochondria in skeletal muscle under conditions
of disuse and reloading following hindlimb unloading. Although apoptosis is an important
signaling process that occurs during unloading, clearly apoptosis is not the only contributor to
muscle wasting, especially in the soleus muscle where muscle loss is typically more severe than
in the plantaris during disuse.

Perspectives and Significance

Although HMB did not prevent skeletal muscle loss induced by HS in aged Fisher
344×Brown Norway rats, HMB did improve muscle recovery following HS and subsequent
reloading. As muscle force should be proportional to the physiological fiber (or muscle) CSA, if
fiber size is better maintained by HMB during the stress of reloading, then there should be a
greater number of cross-bridges which would have the potential to improve force production.
The protective mechanism(s) by which HMB exerts its effects during unloading and improves
recovery following reloading may be due in part to the inhibition of myonuclear apoptosis,
specifically, by suppressing the mitochondrial-caspase signaling pathway. For example, a
reduction in the level of apoptotic signaling during the period of unloading is a possible
mechanism which improves muscle recovery after unloading in HMB treated animals, as a
result of having a greater number of nuclei and therefore improved transcriptional control of
protein synthesis. Nevertheless, it is unlikely that the positive effects of HMB on muscle mass and function under conditions of hindlimb unloading-induced disuse and reloading after hindlimb unloading are solely due to changes in apoptotic signaling. In addition to a reduction in the mitochondrial-associated apoptotic signaling, other possible mechanisms of HMB action include enhancing protein synthesis via the mTOR pathway (24) and/or depression of protein degradation through inhibition of the ubiquitin-proteosome pathway (13,79). Another alternative is that HMB might promote increased activation of satellite cells and/or differentiation of activated satellite cells (42) in response to unloading or reloading to replace nuclei that are eliminated by apoptosis. Additional work is needed to determine if part of the preservation of muscle mass and strength by HMB is on muscle cell proliferation and differentiation, in addition to improving cell survival by reducing apoptosis. In addition, it is possible that if the dose or timing of HMB had differed from that which was used in the current study (e.g., giving HMB only at the onset of reloading) that the responses might differ from the results that we report in this study. Nevertheless, our current data suggest that HMB should be further evaluated and considered as part of a potential therapeutic strategy along with more moderate muscle loading, to prevent muscle loss in aging, and perhaps other conditions of muscle wasting.
Acknowledgements

The authors thank Hua Zhao, Brian Bennett and Eric Scheller for technical assistance provided in this study. We also acknowledge assistance from Karen H. Martin, Ph.D. and the West Virginia University Microscope Imaging Facility, which is supported by the Mary Babb Randolph Cancer Center and NIH grant 5P20RR016440-09. Funding for this project was provided by Abbott Laboratories.
Figure 1. Bodyweight was determined longitudinally in animals at four different time points. The first was prior to giving the animals any dietary intervention (Start). The second (time 0), was after the animals were given either HMB or the vehicle (water) by gavage for 7 days. The final points were after the animals had undergone 14 days of hindlimb suspension (HS) and after 14 days of hindlimb suspension followed by 14 days of reloading (R). Cage control-vehicle treated (CC-Veh) and cage control-HMB (CC-HMB) treated animals did not receive hindlimb suspension but were examined at the same time points as the other animals. Body weight was not significantly different between the HMB and vehicle control groups at any time point. An ANOVA followed by Bonferroni post hoc analyses was used to evaluate the differences between the group means. *, Body weight was significantly lower ($p<0.05$) than the time points before HS for the same treatment. **, Body weight was significantly lower ($p<0.05$) in vehicle and HMB treated groups after both HS and R compared to CC-Veh or CC-HMB groups.

Figure 2. Maximal isometric force production was measured longitudinally in vivo in the plantar flexor muscles before experimental intervention (time 0), after 14 days of hindlimb suspension (HS) and after 14 days of hindlimb suspension followed by 14 days of reloading. The animals received either HMB, or the vehicle (water) daily, for 7 days before time 0 and throughout the experimental period. An ANOVA followed by Bonferroni post hoc analyses was used to evaluate the differences between the group means. †, $p<0.05$, Vehicle vs. HMB. *, Force/body weight was significantly lower ($p<0.05$) compare to points before HS
for the same treatment. **, Force/body weight was significantly lower (p <0.05) in vehicle and HMB treated groups after both HS and R compared to CC-Veh or CC-HMB groups.

Figure 3. Muscle wet weight was obtained in plantaris (A) and soleus (C) muscles of control animals for the hindlimb suspension group (HS Con), the recovery group (R Con), and in experimental animals after 14 days of hindlimb suspension (HS) or after 14 days of hindlimb suspension followed by 14 days of reloading (R). The ratio of muscle wet weight to bodyweight is presented for the plantaris (B) and the soleus (D) muscles of the aged animals after each condition. The animals received HMB or the vehicle (water) daily by gavage, for a total of 21 days (HS Con and HS) or for 32 days (R Con and R). An ANOVA followed by Bonferroni post hoc analyses was used to evaluate the differences between the group means. *, p<0.05, Vehicle vs. HMB within the same condition; †, p<0.05 HS or R vs. control animals for that experimental condition.

Figure 4. Fiber cross sectional area (CSA) was obtained by planimetry in the plantaris (A) and the soleus (C) muscles of control animals for the hindlimb suspension group (HS Con), the recovery group (R Con), and in experimental animals after 14 days of hindlimb suspension (HS), or after 14 days of hindlimb suspension followed by 14 days of reloading (R). The animals received HMB or the vehicle (water) daily by gavage, for 7 days of pretreatment, followed by 14 days (HS Con and HS) or for 28 days (R Con and R) of the respective interventions. The fiber area-fiber frequency distribution is shown in 100 µm² bin widths for the plantaris (B) and soleus (D) muscles of animals in the recovery (R) group. An ANOVA followed by Bonferroni post hoc analyses was used to evaluate the differences between the
group means. *, p<0.05, Vehicle vs. HMB within the same condition; †, p<0.05.

HS or R vs. control animals for that experimental condition.

Figure 5.  

A. Top row. Representative tissue sections from the plantaris muscle, with fluorescent staining for TUNEL (green) to identify apoptotic nuclei in control and hindlimb suspended muscles. DAPI identified all nuclei (blue). The basal lamina (red) was identified to confirm that the TUNEL positive nuclei were myonuclei. The conditions were vehicle control (Vehicle Con), HMB control (HMB Con), vehicle after 14 days of HS (Vehicle HS), and HMB after 14 days of HS (HMB HS). The arrows show TUNEL positive nuclei lying below or immediately adjacent to the basal lamina of the muscle fibers. Bottom row. A higher magnification showing the individual markers for TUNEL, laminin, DAPI and the combined images. The arrows show TUNEL positive nuclei lying below or immediately adjacent to the basal lamina of the muscle fibers.

B. Top row. Representative tissue sections from the plantaris muscle, with fluorescent staining for TUNEL (green) to identify apoptotic nuclei in control and reloaded muscles. DAPI identified all nuclei (blue). The basal lamina (red) was identified to confirm that the TUNEL positive nuclei were myonuclei. The conditions were vehicle control (Vehicle Con), HMB control (HMB Con), vehicle after 14 days of hindlimb suspension followed by 14 days of reloading (Vehicle R), and HMB after 14 days of hindlimb suspension following 14 days of reloading (HMB R). The arrows show TUNEL positive nuclei lying below or immediately adjacent to the basal lamina of the muscle fibers. Bottom row. A higher magnification showing the individual markers for TUNEL, laminin, DAPI and the
combined images. The arrows show TUNEL positive nuclei lying below or immediately adjacent to the basal lamina of the muscle fibers.

C. The apoptotic index was calculated from tissue cross sections of the plantaris muscle by determining the ratio of TUNEL positive nuclei to total nuclei in plantaris muscles of control animals for the hindlimb suspension group (HS Con), the recovery group (R Con), and in experimental animals after 14 days of hindlimb suspension (HS) or after 14 days of hindlimb suspension followed by 14 days of reloading (R). Only nuclei that were directly below or touching the basal lamina were counted. The animals received HMB or the vehicle (water) daily by gavage, for a total of 21 days (HS Con and HS) or for 32 days (R Con and R). An ANOVA followed by Bonferroni post hoc analyses was used to evaluate the differences between the group means. *, p<0.05, Vehicle vs. HMB within the same condition; †, p<0.05, HS or R vs. control animals for that experimental condition.

Figure 6. A. Top row. Representative tissue sections from the soleus muscle, with fluorescent staining for TUNEL (green) to identify apoptotic nuclei in control and hindlimb suspended muscles. DAPI identified all nuclei (blue). The basal lamina (red) was identified to confirm that the TUNEL positive nuclei were myonuclei. The conditions were vehicle control (Vehicle Con), HMB control (HMB Con), vehicle after 14 days of HS (Vehicle HS), and HMB after 14 days of HS (HMB HS). The arrows show TUNEL positive nuclei lying below or immediately adjacent to the basal lamina of the muscle fibers. Bottom row. A higher magnification showing the individual markers for TUNEL, laminin, DAPI and the
combined images. The arrows show TUNEL positive nuclei lying below or immediately adjacent to the basal lamina of the muscle fibers.

**B.** Top row. Representative tissue sections from the soleus muscle, with fluorescent staining for TUNEL (green) to identify apoptotic nuclei in control and reloaded muscles. DAPI identified all nuclei (blue). The basal lamina (red) was identified to confirm that the TUNEL positive nuclei were myonuclei. The conditions were vehicle control (Vehicle Con), HMB control (HMB Con), vehicle after 14 days of hindlimb suspension followed by 14 days of reloading (Vehicle R), and HMB after 14 days of hindlimb suspension following 14 days of reloading (HMB R). The arrows show TUNEL positive nuclei lying below or immediately adjacent to the basal lamina of the muscle fibers. Bottom row. A higher magnification showing the individual markers for TUNEL, laminin, DAPI and the combined images. The arrows show TUNEL positive nuclei lying below or immediately adjacent to the basal lamina of the muscle fibers.

**C.** The apoptotic index was calculated from tissue cross sections of the soleus muscle by determining the number ratio of TUNEL positive nuclei to total nuclei in soleus muscles of control animals for the hindlimb suspension group (HS Con), the recovery group (R Con), and in experimental animals after 14 days of hindlimb suspension (HS) or after 14 days of hindlimb suspension followed by 14 days of reloading (R). Only nuclei that were directly below or touching the basal lamina were counted. The animals received either HMB or the vehicle (water) daily by gavage, for a total of 21 days (HS Con and HS) or for 32 days (R Con and R). An ANOVA followed by Bonferroni post hoc analyses was used to evaluate the differences between the group means. *, p<0.05, Vehicle vs. HMB.
within the same condition; †, \( p<0.05 \), HS or R vs. control animals for that experimental condition.

**Figure 7.** Bax protein abundance was determined by western blot analysis in the plantaris (A) and soleus (B) muscles of rats under control, hindlimb suspension, or reloading conditions. The groups include: controls for the hindlimb suspension group (HS Con), controls for the recovery group (R Con), and in experimental animals after 14 days of hindlimb suspension (HS) or after 14 days of hindlimb suspension followed by 14 days of reloading (R). The animals received HMB or the vehicle (water) daily by gavage, for a total of 21 days (HS Con and HS) or for 32 days (R Con and R). Eight animals were in each diet and experimental group. \( \alpha \)-tubulin was used as a loading control. The data were normalized to \( \alpha \)-tubulin and were expressed as mean ± SD. An ANOVA followed by Bonferroni post hoc analyses was used to evaluate the differences between the group means. *, \( p<0.05 \), Vehicle vs. HMB within the same condition; †, \( p<0.05 \), HS or R vs. control animals for that experimental condition.

**Figure 8.** Cleaved caspase-9 protein abundance was determined by western blot analysis in the plantaris (A) and soleus (B) muscles of rats under control, hindlimb suspension, or reloading conditions. The groups include: controls for the hindlimb suspension group (HS Con), controls for the recovery group (R Con), and in experimental animals after 14 days of hindlimb suspension (HS) or after 14 days of hindlimb suspension followed by 14 days of reloading (R). The animals received HMB or the vehicle (water) daily by gavage, for a total of 21 days (HS Con and HS) or for 32 days (R Con and R). Eight animals were in each diet and experimental group. \( \alpha \)-tubulin was used as a loading control. The data were
normalized to α-tubulin and were expressed as mean ± SD. An ANOVA followed by Bonferroni post hoc analyses was used to evaluate the differences between the group means. *, p<0.05, Vehicle vs. HMB within the same condition; †, p<0.05, HS or R vs. control animals for that experimental condition.

Figure 9. Cleaved caspase-9 protein abundance was determined by western blot analysis in the plantaris (A) and soleus (B) muscles of rats under control, hindlimb suspension, or reloading conditions. The groups include: controls for the hindlimb suspension group (HS Con), controls for the recovery group (R Con), and in experimental animals after 14 days of hindlimb suspension (HS) or after 14 days of hindlimb suspension followed by 14 days of reloading (R). The animals received HMB or the vehicle (water) daily by gavage, for a total of 21 days (HS Con and HS) or for 32 days (R Con and R). Eight animals were in each diet and experimental group. α-tubulin was used as a loading control. The data were normalized to α-tubulin and were expressed as mean ± SD. An ANOVA followed by Bonferroni post hoc analyses was used to evaluate the differences between the group means. *, p<0.05, Vehicle vs. HMB within the same condition; †, p<0.05, HS or R vs. control animals for that experimental condition.

Figure 10. Bcl-2 protein abundance was determined by western blot analysis in the plantaris (A) and soleus (B) muscles of rats under control, hindlimb suspension, or reloading conditions. The groups include: controls for the hindlimb suspension group (HS Con), controls for the recovery group (R Con), and in experimental animals after 14 days of hindlimb suspension (HS) or after 14 days of hindlimb suspension followed by 14 days of reloading (R). The animals received HMB or
the vehicle (water) daily by gavage, for a total of 21 days (HS Con and HS) or for 32 days (R Con and R). Eight animals were in each diet and experimental group. α-tubulin was used as a loading control. The data were normalized to α-tubulin and were expressed as mean ± SD. An ANOVA followed by Bonferroni post hoc analyses was used to evaluate the differences between the group means. †, $p<0.05$, HS or R vs. control animals for that experimental condition.
Table S1. **Bodyweight for aged rats.** The bodyweights of cage control rats, or rats that underwent 14 days of HS or 14 days of recovery following HS are presented as mean ± SD. Cage control-vehicle treated (CC-Veh) and cage control-HMB (CC-HMB) treated animals did not receive hindlimb suspension but were examined at the same time points as the other animals.

Table S2. **Isometric force/bodyweight for aged rats.** The plantarflexor isometric force/bodyweight ratio of rats is reported in each group as Newtons of force/gram bodyweight (mean ± SD). The conditions include: after cage control conditions, after 14 days of HS or 14 days of recovery following HS. Cage control-vehicle treated (CC-Veh) and cage control-HMB (CC-HMB) treated animals did not receive hindlimb suspension but were examined at the same time points as the other animals.

Table S3. **Gastrocnemius muscle wet weight for aged animals.** Gastrocnemius muscle wet weight data (mean ± SD) are summarized from aged animals that were used for the current study under control conditions, or after 14 days of HS or 14 days of recovery following HS. Cage control-vehicle treated (CC-Veh) and cage control-HMB (CC-HMB) treated animals did not receive hindlimb suspension but were examined at the same time points as the other animals. Gastrocnemius wet weight was not significantly reduced by HS or HS+ recovery.
Table S4. **Bodyweight for young adult rats.** The bodyweight of young adult rats (12 months of age) were obtained before HS, after 14 days of HS or after 14 days of recovery following HS. The dietary and control conditions were the same as used for the aged animals. The conditions are after cage control conditions, after 14 days of HS or 14 days of recovery following HS. Cage control-vehicle treated (CC-Veh) and cage control-HMB (CC-HMB) treated animals did not receive hindlimb suspension but were examined at the same time points as the other animals. Values are means ± SD. Time 0, before hindlimb suspension; 14d HS, 14 days after hindlimb suspension; Recover, after 14 days of HS followed by 14 days of reloading. § *p*<0.05, compared to Pre-HS.* *p*<0.05, compared to Post-HS.

Table S5. **Isometric force/bodyweight for young adult rats.** The plantarflexor isometric force/bodyweight ratio for young adult rats (12 months) in each group is reported as Newtons of force/gram bodyweight. The conditions are: after cage control conditions, after 14 days of HS or 14 days of recovery following HS. Cage control-vehicle treated (CC-Veh) and cage control-HMB (CC-HMB) treated animals did not receive hindlimb suspension but were examined at the same time points as the other animals. Values are means ± SD. Time 0, before hindlimb suspension; 14d HS, 14 days after hindlimb suspension; Recover, after 14 days of HS followed by 14 days of reloading.

Figure S1. **Muscle wet weight data for young animals subjected to hindlimb suspension or after recovery following unloading.** Muscle wet weight was obtained in plantaris (A) and soleus (C) muscles of young adult rats (12 months of age) control animals for the hindlimb suspension group (HS Con), the recovery
group (R Con), and in experimental animals after 14 days of hindlimb suspension (HS) or after 14 days of hindlimb suspension followed by 14 days of reloading (R). The ratio of muscle wet weight to bodyweight is presented for the plantaris (B) and the soleus (D) muscles of the aged animals after each condition. The animals received HMB or the vehicle (water) daily by gavage, for a total of 21 days (HS Con and HS) or for 32 days (R Con and R). An ANOVA followed by Bonferroni post hoc analyses was used to evaluate the differences between the group mean. †, $p<0.05$, HS vs. R animals or control animals for that experimental condition. Six animals were examined in each group. There was no effect of HMB on muscle wet weight responses to loading or recovery.

**Figure S2.** The apoptotic index. The apoptotic index was calculated from tissue cross sections of the plantaris muscle (A) and the soleus muscle (B) of young adult FBN rats (12 months of age) by determining the number ratio of TUNEL positive nuclei to total nuclei in tissue cross sections. The muscle groups were: the cage control animals for the hindlimb suspension group (HS Con), the cage control animals for the recovery group (R Con), and in experimental animals after 14 days of hindlimb suspension (HS) or after 14 days of hindlimb suspension followed by 14 days of reloading (R). Only nuclei that were directly below or touching the basal lamina were counted. The animals received either HMB or the vehicle (water) daily by gavage, for a total of 21 days (HS Con and HS) or for 32 days (R Con and R). An ANOVA followed by Bonferroni post hoc analyses was used to evaluate the differences between the group means. †, $p<0.05$, HS vs. R animals or control animals for that experimental condition. Six animals were examined in each group. The data are presented as mean ± SD.
**Figure S3.** Apoptotic signaling proteins in the plantaris muscle of young adult rats.

The protein abundance was determined by western blots in the plantaris muscle of young adult (12 months of age) FBN rats for Bax (A), Bcl-2 (B), cleaved caspase-9 (C), and cleaved caspase-3 (D). The groups include: controls for the hindlimb suspension group (HS Con), controls for the recovery group (R Con), and in experimental animals after 14 days of hindlimb suspension (HS) or after 14 days of hindlimb suspension followed by 14 days of reloading (R). The animals received HMB or the vehicle (water) daily by gavage, for a total of 21 days (HS Con and HS) or for 32 days (R Con and R). Six animals were examined in each group. α-tubulin was used as a loading control. The data were normalized to α-tubulin and were expressed as mean ± SD. An ANOVA followed by Bonferroni post hoc analyses was used to evaluate the differences between the group means. †, *p*<0.05, HS vs. R animals or control animals for that experimental condition. All apoptotic markers had returned to control levels in the R group. HMB had no effect on apoptotic signaling in the plantaris muscles of young adult rats.

**Figure S4.** Apoptotic signaling proteins in the soleus muscle of young adult rats. The protein abundance was determined by western blots in the soleus muscle of young adult (12 months of age) FBN rats for Bax (A), Bcl-2 (B), cleaved caspase-9 (C), and cleaved caspase-3 (D). The groups include: controls for the hindlimb suspension group (HS Con), controls for the recovery group (R Con), and in experimental animals after 14 days of hindlimb suspension (HS) or after 14 days of hindlimb suspension followed by 14 days of reloading (R). The animals received HMB or the vehicle (water) daily by gavage, for a total of 21 days (HS Con and HS) or for 32 days (R Con and R). Six animals were in each
diet and experimental group. α-tubulin was used as a loading control. The data were normalized to α-tubulin and were expressed as mean ± SD. An ANOVA followed by Bonferroni post hoc analyses was used to evaluate the differences between the group means. †, p<0.05, HS vs. R animals or control animals for that experimental condition. All apoptotic markers had returned to control levels in the R group. HMB had no effect on apoptotic signaling in the plantaris muscles of young adult rats.
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66. **Ryan MJ, Dudash HJ, Docherty M, Geronilla KB, Baker BA, Haff GG, Cutlip RG and Alway SE.** Vitamin E and C supplementation reduces oxidative stress, improves


87. van Someren KA, Edwards AJ and Howatson G. Supplementation with beta-hydroxy-beta-methylbutyrate (HMB) and alpha-ketoisocaproic acid (KIC) reduces signs and


Figure 1

[Graph showing body weight changes over time for different groups: CC-Veh, CC-HMB, Vehicle, HMB. The graph indicates a decrease in body weight with significant differences marked by asterisks (* and **).]
Figure 2

Isometric Force

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Force/body weight (N/Kg)

- * indicates significant difference from baseline.
- ** indicates highly significant difference from baseline.
Figure 3

A. Plantaris Muscle Wet Weight
B. Plantaris Muscle Wet Weight/Bodyweight
C. Soleus Muscle Wet Weight
D. Soleus Muscle Wet Weight/Bodyweight
Figure 4

A

Plantaris

B

Plantaris: Recovery

C

Soleus

D

Soleus: Recovery
Figure 5

A. TUNEL Staining in Plantaris Muscles After HS
B. TUNEL Staining in Plantaris Muscles After Reloading
Figure 5

C  TUNEL Positive Nuclei in the Plantaris Muscle

![Graph showing TUNEL positive nuclei in the Plantaris Muscle. The x-axis represents groups: HS Con, R Con, HS, R. The y-axis represents apoptotic index (%). The graph compares Vehicle and HMB conditions.](image)
Figure 6

A. TUNEL Staining in Soleus Muscles After HS
B. TUNEL Staining in Soleus After Reloading
Figure 6

C  TUNEL Positive Nuclei in the Soleus Muscle

Apoptotic Index (%)
Figure 7

A

Bax Protein Content

Plantaris

Arbitrary Units (×10^3)

Vehicle  HMB

Bax α Tubulin

HS Con  R Con  HS  R

B

Plantaris

Arbitrary Units (×10^3)

Vehicle  HMB

Bax α Tubulin

HS Con  R Con  HS  R

†  *
Figure 8

Cleaved Caspase-9

A

Plantaris

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>HMB</th>
</tr>
</thead>
<tbody>
<tr>
<td>HS Con</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>R Con</td>
<td>15</td>
<td>*</td>
</tr>
<tr>
<td>HS</td>
<td>20</td>
<td>†</td>
</tr>
<tr>
<td>R</td>
<td>25</td>
<td>†‡</td>
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Soleus

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>HMB</th>
</tr>
</thead>
<tbody>
<tr>
<td>HS Con</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>R Con</td>
<td>20</td>
<td>*</td>
</tr>
<tr>
<td>HS</td>
<td>25</td>
<td>†</td>
</tr>
<tr>
<td>R</td>
<td>30</td>
<td>†‡</td>
</tr>
</tbody>
</table>

Arbitrary Units (x10^3)

α Tubulin

CCS-9
Figure 9.

A

Cleaved Caspase-3

Plantaris

B

Soleus

Arbitrary Units (x10^4)

CCS-3

α Tubulin

HS Con R Con HS R

Vehicle HMB

* †
Figure 10

A

Bcl-2

Plantaris

\[ \text{Arbitrary Units (x10^3)} \]

\[ \begin{array}{c}
\text{Bcl-2} \\
\alpha\text{-Tubulin}
\end{array} \]

\[ \begin{array}{c}
\text{HS Con} \\
\text{R Con} \\
\text{HS} \\
\text{R}
\end{array} \]

B

Soleus

\[ \text{Arbitrary Units (x10^3)} \]

\[ \begin{array}{c}
\text{Bcl-2} \\
\alpha\text{-Tubulin}
\end{array} \]

\[ \begin{array}{c}
\text{HS Con.} \\
\text{R Con} \\
\text{HS} \\
\text{R}
\end{array} \]