Attenuation of Proteasome-Induced Proteolysis in Skeletal Muscle by β-Hydroxy-β-Methylbutyrate in Cancer-Induced Muscle Loss

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Abstract

Loss of skeletal muscle is an important determinant of survival in patients with cancer-induced weight loss. The effect of the leucine metabolite β-hydroxy-β-methylbutyrate (HMB) on the reduction of body weight loss and protein degradation in the MAC16 model of cancer-induced weight loss has been compared with that of eicosapentaenoic acid (EPA), a recognized inhibitor of protein degradation. HMB was found to attenuate the development of weight loss at a dose greater than 0.125 g/kg accompanied by a small reduction in tumor growth rate. When EPA was used at a suboptimal dose level (0.6 g/kg) the combination with HMB seemed to enhance the anticachectic effect. Both treatments caused an increase in the wet weight of soleus muscle and a reduction in protein degradation, although there did not seem to be a synergistic effect of the combination. Proteasome activity, determined by the "chymotrypsin-like" enzyme activity, was attenuated by both HMB and EPA. Protein expression of the 20S α or β subunits was reduced by at least 50%, as were the ATPase subunits MSS1 and p42 of the 19S proteasome regulatory subunit. This was accompanied by a reduction in the expression of $E2_{14k}$ ubiquitin-conjugating enzyme. The combination of EPA and HMB was at least as effective or more effective than either treatment alone. Attenuation of proteasome expression was reflected as a reduction in protein degradation in gastrocnemius muscle of cachectic mice treated with HMB. In addition, HMB produced a significant stimulation of protein synthesis in skeletal muscle. These results suggest that HMB preserves lean body mass and attenuates protein degradation through down-regulation of the increased expression of key regulatory components of the ubiquitin-proteasome proteolytic pathway, together with stimulation of protein synthesis. (Cancer Res 2005; 65(1): 277-83)

Introduction

Weight loss is common in patients with carcinomas of the lung and gastrointestinal tract (1), resulting in a massive loss of both body fat and muscle protein, whereas non–muscle protein remains unaffected (2). Although loss of body fat is important in terms of energy reserves, it is loss of skeletal muscle protein that results in immobility and, eventually, impairment of respiratory muscle function (3), leading to death from hypostatic pneumonia. Although cachexia is frequently accompanied by anorexia, nutritional supplementation alone is unable to maintain stable body weight and any weight that is gained is due to an increase in adipose tissue and water rather than lean body mass (4). The same is true for appetite stimulants, such as megestrol acetate (5) and medroxyprogesterone acetate (6), suggesting that loss of lean body mass is due to factors other than energy insufficiency.

Skeletal muscle mass is a balance between the rate of protein synthesis and the rate of degradation. Patients with cancer-induced weight loss show a depression of protein synthesis in skeletal muscle (7) and an increase in protein degradation, which is reflected in an increased expression of the ubiquitin-proteasome proteolytic pathway, the major determinant of protein degradation. Thus, skeletal muscle from cachectic cancer patients shows increased expression of mRNA for both ubiquitin (8) and proteasome subunits (9), whereas proteasome proteolytic activity increased in parallel with ubiquitin expression (8). The inability of anabolic stimuli to increase lean body mass in cachectic patients suggests that protein degradation must be attenuated before muscle mass can increase. Eicosapentaenoic acid (EPA), down-regulates the increased expression of the ubiquitin-proteasome proteolytic pathway in the skeletal muscle of cachectic mice (10) and has been shown to stabilize body weight in cachectic patients with pancreatic cancer (11). When patients consumed an energy-dense supplement containing 32 g protein and 2 g EPA, body weight increased, and this was attributed solely to an increase in lean body mass (12).

β-Hydroxy-β-methylbutyrate (HMB) is a metabolite of leucine formed by transamination to α-ketoisocaproate in muscle followed by oxidation of the α-ketoisocaproate in the cytosol of the liver, and possibly other tissues, to give HMB (13). Both leucine and α-ketoisocaproate have been proposed to decrease nitrogen and protein loss and the effect seems to be due to the production of HMB (14). A recent study (15) showed a mixture of HMB, arginine, and glutamine to be effective in increasing body weight in weight-losing patients with advanced (stage IV) cancer. Moreover, the increase in body weight was attributed to an increase in fat-free mass, as observed with EPA (12).

This study evaluates the effect of HMB, in comparison with EPA or the combination, on weight loss induced by the MAC16 tumor and the mechanisms involved. Weight loss induced by the MAC16 tumor does not involve anorexia (16) or the cytokine tumor necrosis factor-α or interleukin 6 (17). Proteasome functional activity and protein levels of proteasome subunits were chosen to measure expression of the ubiquitin-proteasome pathway because of reports (18, 19) that in various cells elevated concentrations of mRNA of proteasome subunits were not found to be accompanied by increased concentrations or activities of proteasomes.

Materials and Methods

Materials. EPA (98% as free acid) was purchased from Biomol Research Laboratories Inc. (Plymouth Meeting, PA). HMB (as the calcium

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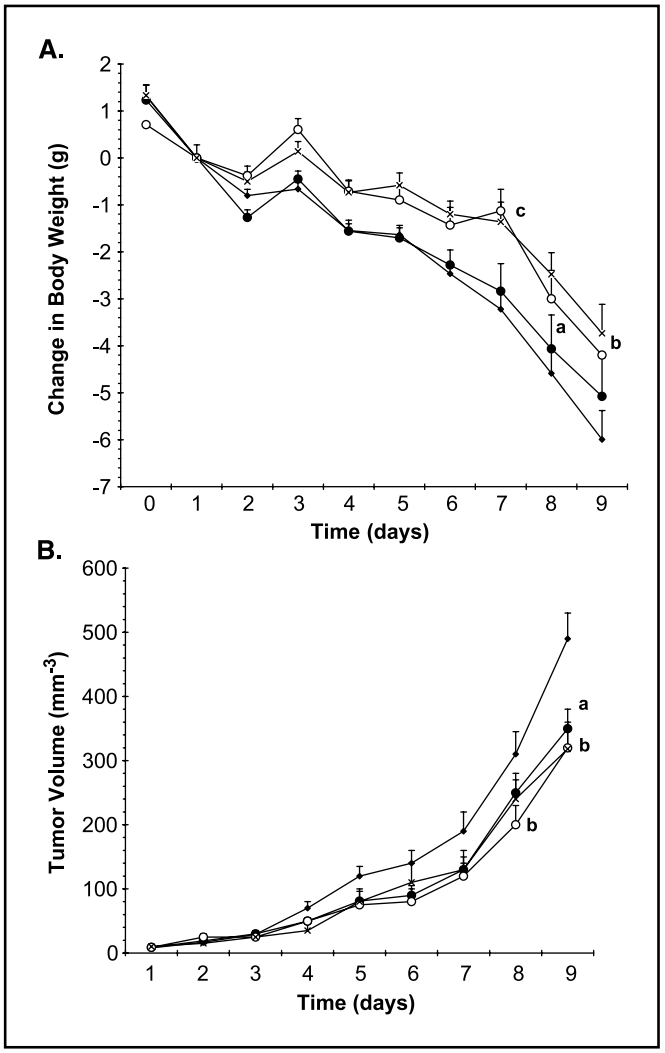

Figure 1. Dose-response curves for the effect of HMB on body weight (*A*) expressed as change in body weight (g) and tumor volume (mm^3) (*B*) in mice bearing the MAC16 tumor. This study was initiated (day 1) 9 days after tumor transplantation before weight loss was apparent. HMB (in PBS) was given p.o. by gavage on a daily regimen at a concentration of 0.05 (●), 0.125 (○), and 0.25 g/kg (×). Control mice received PBS alone (♦). *Points,* mean ($n = 20$); *bars,* SE. Differences from the control group: a, $P < 0.05$; b, $P < 0.01$, and c, $P < 0.005$.

salt) was obtained from Organic Technologies Inc. (Coshocton, OH). Mouse monoclonal antibodies to 20S proteasome subunits α 1, 2, 3, 5, 6, and 7 (clone MCP 231), 20S proteasome subunit β3 (HC10), 19S regulator ATPase subunit Rpt 1 (S7, Mss1; clone MSS1-104), and 19S regulator ATPase subunit Rpt 4 (S106, p42; clone p42-23) were purchased from Affiniti Research Products (Exeter, United Kingdom). Rabbit polyclonal antisera to ubiquitin-conjugating enzyme E2 (anti-UBC2 antibody) was a gift from Dr. Simon Wing (McGill University, Montreal, Quebec, Canada). Peroxidase-conjugated goat anti-rabbit and rabbit anti-mouse secondary antibodies were from Dako Ltd. (Cambridge, United Kingdom).

Animals. Pure strain male NMRI mice (average weight 25 g) were obtained from our own inbred colony and were transplanted with fragments of the MAC16 tumor s.c. into the flank by means of a trochar, selecting from donor animals with established weight loss (16). Transplanted animals were given a rat and mouse breeding diet (Special Diet Services, Witham, United Kingdom) and water *ad libitum*, and weight loss was evident 10 to 12 days after tumor implantation. Animals just prior to the development of weight loss were randomized to receive daily EPA (in olive oil), HMB (in PBS), or the combination as described in the figure legends given p.o. by gavage, whereas control animals received either olive oil or PBS. All groups contained a minimum of six mice. Tumor volume, body weight, and food and water intake were monitored daily. Animals were terminated by cervical dislocation when the body weight loss reached 25%, and all studies were conducted according to the United Kingdom Co-ordinating Committee of Cancer Research guidelines for the care and use of laboratory animals. The soleus muscles were quickly dissected, together with intact tendons, and maintained in isotonic ice-cold saline before determination of protein degradation. Body composition analysis was determined as previously described (20).

Determination of Muscle Protein Degradation. Freshly dissected soleus muscles were fixed via the tendons to aluminum wire supports, under tension, at approximately resting length to prevent muscle shortening and preincubated for 45 minutes in 3 mL of oxygenated (95% oxygen/5% carbon dioxide) Krebs-Henseleit bicarbonate buffer (pH 7.4) containing 5 mmol/L glucose and 0.5 mmol/L cycloheximide. Protein degradation was determined by the release of tyrosine over a 2-hour period (21).

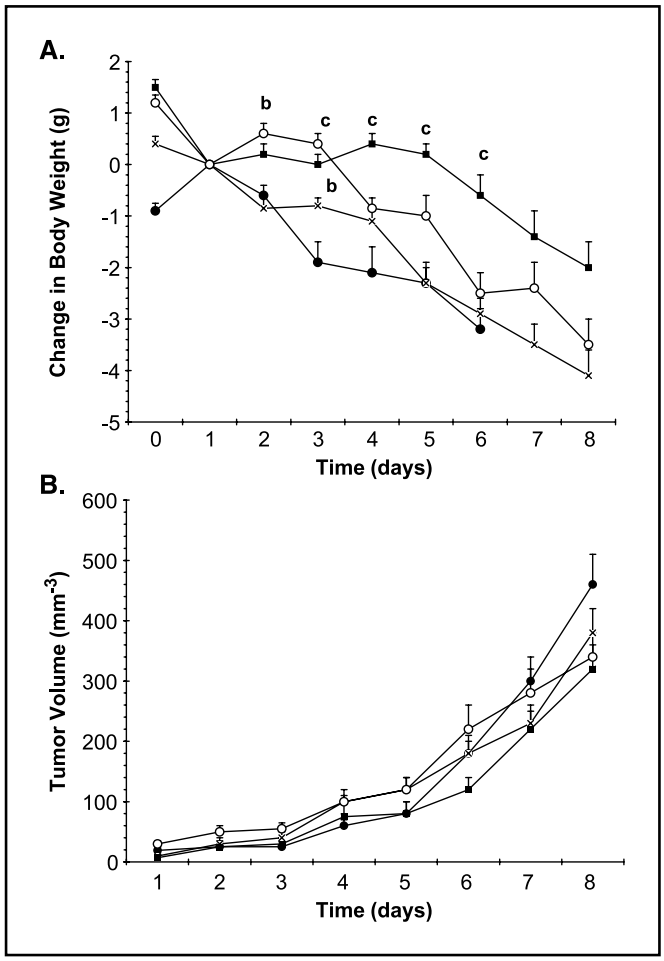

Figure 2. Effect of 0.25 g/kg HMB (■), 0.6 g/kg EPA (×), and the combination (○) together with PBS controls (●) on body weight expressed as change on body weight (g) (*A*) and tumor volume (mm^3) (*B*) of mice bearing the MAC16 tumor. The study was initiated 9 days after tumor transplantation (day 1). All agents were given p.o. by gavage on a daily regimen and the dosing of EPA and HMB was separated by 2 hours. *Points,* mean ($n = 20$); *bars,* SE. Differences from the control group: a, $P < 0.05$; b, $P < 0.01$, or c, $P < 0.005$.

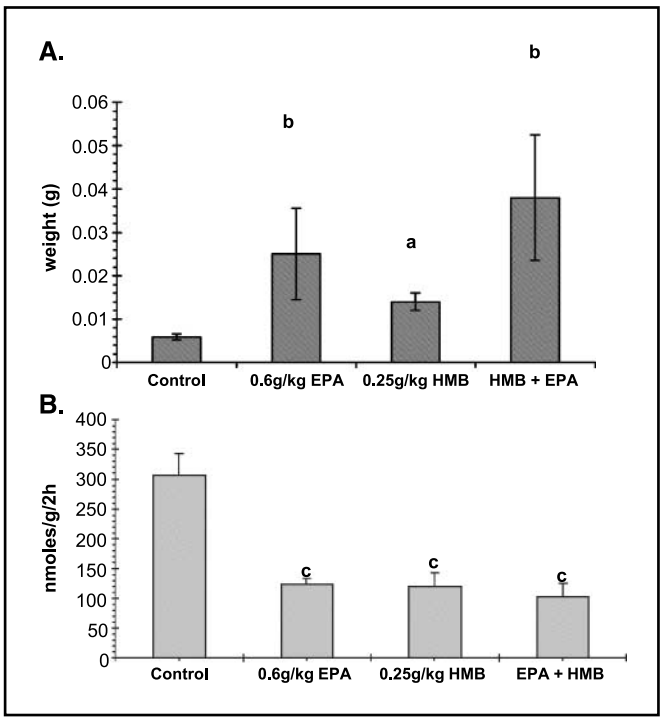

Figure 3. Wet weight of soleus muscles (*A*) and rate of protein degradation in soleus muscle expressed as nanomoles of tyrosine released per gram of muscle over 2 hours (*B*) of mice bearing the MAC16 tumor and treated with either 0.6 g/kg EPA, 0.25 g/kg HMB, or the combination for 3 days. *Columns,* mean ($n = 6$); *bars,* SE. Differences from the PBS control group: a, $P < 0.05$, b, $P < 0.01$, or c, $P < 0.005$.

Measurement of Proteasome Activity. Functional proteasome activity was determined by measuring the "chymotrypsin-like" enzyme activity, the predominant proteolytic activity of the β subunits of the proteasome according to the method of Orino et al. (22). Muscles were rinsed with ice-cold PBS, minced, and sonicated in 20 mmol/L Tris-HCl (pH 7.5), 2 mmol/L ATP, 5 mmol/L $MgCl_2$, and 1 mmol/L DTT. The sonicate was then centrifuged for 10 minutes at 18,000 × *g*, at 4C and the supernatant was used to determine chymotrypsin-like enzyme activity by the release of aminomethyl coumarin (AMC) from the fluorogenic substrate succinyl-LLVY-AMC. Activity was measured in the absence and presence of the specific proteasome inhibitor lactacystin (10 M). Only lactacystin suppressible activity was considered to be proteasome specific.

Protein Synthesis and Degradation in Gastrocnemius Muscle. The method for the determination of protein synthesis and degradation in gastrocnemius muscle has been previously described (23). Briefly protein synthesis was measured by the incorporation of L-[4-^{3}H]phenylalanine during a 2-hour period in which isolated gastrocnemius muscles were incubated at 37°C in RPMI 1640 without phenol red and saturated with O_2/CO_2 (19:1). After incubation muscles were rinsed in nonradioactive medium, blotted dry, and homogenized in 4 mL 2% perchloric acid. The rate of protein synthesis was calculated by dividing the protein-bound radioactivity by the acid-soluble (unbound) material.

For protein degradation assays animals from the same group as used to measure protein synthesis were given i.p. 0.4 mmol/L L-[4-^{3}H]phenylalanine in PBS (100 μl) 24 hours prior to the assay. Isolated gastrocnemius muscles were extensively washed with PBS and RPMI 1640 before measuring the release of radioactivity into RPMI 1640 over a 2-hour period. The protein-bound activity was determined by homogenizing the muscles in 2% perchloric acid and determining the non–acid-soluble radioactivity (radioactivity in the precipitate). The rate of protein degradation was calculated by dividing the amount of [^{3}H]phenylalanine radioactivity released into the incubation medium during the 2-hour incubation period by the specific activity of protein-bound [^{3}H] phenylalanine.

Western Blot Analysis. Samples of soleus muscle cytosolic protein (2 to 5 μg) obtained from the above assay were resolved on 10% SDS-PAGE and transferred to 0.45-μm nitrocellulose membrane (Hybond, Amersham Life Science Products, Bucks, United Kingdom), which had been blocked with 5% Marvel in PBS. The primary antibodies for MSS1 and p42 were used at a dilution of 1:5,000, for 20S proteasome α subunits at 1:1500, and for β subunits at 1:1000, whereas the antibody for E_{214k} was used at a dilution of 1:500. The secondary antibodies were used at a dilution of 1:2000. Incubation was carried out for 2 hours at room temperature and developed by enhanced chemiluminescence (Amersham, Bucks, United Kingdom).

Statistical Analysis. Differences in means between groups was determined by one-way ANOVA followed by Tukey-Kramer multiple comparison test.

Results

A dose-response relationship of HMB alone on weight loss and tumor growth rate in mice bearing the MAC16 tumor is shown in Fig. 1. Doses of HMB >0.125 g/kg caused a significant reduction in weight loss (Fig. 1*A*) and this was accompanied by a small but significant reduction in tumor growth rate (Fig. 1*B*). Attenuation of weight loss was not accompanied by an alteration in food and water intake. A dose level of 0.25 g/kg was chosen for all subsequent experiments. The effect of HMB, EPA and the combination of HMB and EPA on weight loss in MAC16 cachectic tumor-bearing mice is shown in Fig. 2*A*. A suboptimal dose of EPA was chosen to investigate interactions with HMB. Weight loss was attenuated by EPA, whereas HMB and the combination seemed to be slightly less effective than EPA alone. There was no significant effect of any treatment on tumor volume over the same period (Fig. 2*B*). All treatments caused a significant increase in soleus wet muscle weight (Fig. 3*A*), although there seems to be considerable variation between muscle weights within the EPA and combination treatment groups due to the relatively small size of these muscles (<100 g of muscle). The protein degradation in these muscles was measured by tyrosine released per gram of muscle over 2 hours, taking into consideration the variation in muscle sizes (Fig. 3*B*). At the doses chosen HMB was as effective as EPA and there did not seem to be a synergistic effect of the combination. Body composition analysis (Table 1) indicated that HMB caused a significant increase in the nonfat carcass mass without an effect on adipose tissue.

Proteasome expression has been shown to be elevated in gastrocnemius muscles of mice bearing the MAC16 tumor and this increased gene expression has been shown to be attenuated by EPA

Table 1. Body composition analysis of mice bearing the MAC16 tumor treated with HMB for 5 days

HMB (g/kg)	Water (%)	Fat (%)	Nonfat (%)
0	70.8 ± 2.7	3.8 ± 1.3	25.4 ± 1.9
0.125	65.5 ± 0.9	3.3 ± 1.6	31.2 ± 1.5*
0.25	66.5 ± 2.2	4.4 ± 1.3	29.2 ± 1.9*
0.5	67.2 ± 2.3	3.8 ± 1.2	29.0 ± 1.4*

*$P < 0.01$ from control group receiving 0 g/kg HMB.

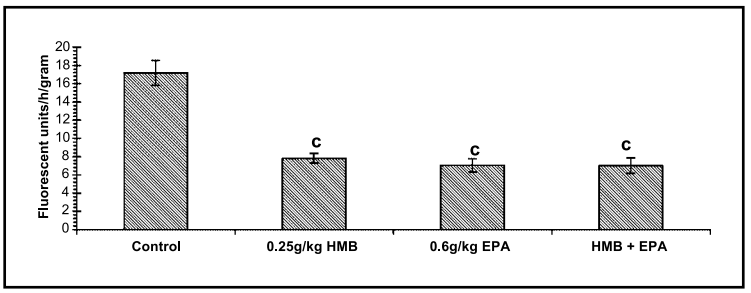

Figure 4. Effect of HMB and EPA on proteasome functional activity, determined as the chymotrypsin-like enzyme activity in gastrocnemius muscle of mice bearing the MAC16 tumor and treated for 3 days. *Columns,* mean fluorometric activity per gram of protein over 1 hour expressed in arbitrary fluorescence units ($n = 6$); *bars,* SE. Differences from control: c, $P < 0.005$.

(10). The results in Fig. 4 show that functional proteasome activity, as determined by chymotrypsin-like enzyme activity, was attenuated by HMB to the same extent as EPA at the doses chosen, and that the combination of HMB and EPA did not produce a further depression in activity. Protein expression of proteasome subunits was analyzed by Western blotting of cellular supernatants. Expression of 20S proteasome α subunits, the structural units of the proteasome, was attenuated by both HMB and EPA, and there was some indication of a further decrease of band 2 for the combination (Fig. 5*B*). Expression of the 20S proteasome subunits, the catalytic subunits of the proteasome, were also attenuated by HMB and EPA, and with the combination attenuation was significant unlike either agent alone (Fig. 5*C* and *D*). Expression of MSS1, an ATPase subunit of the 19S proteasome regulatory complex, is shown in Fig. 6*A*. Both HMB and EPA attenuated MSS1 expression, but the combination did not seem to produce a further reduction. Similar results were obtained with p42, another ATPase subunit of the 19S regulator that promotes ATP-dependent association of the 20S proteasome with the 19S regulator to form the 26S proteasome (Fig. 6*C*). Again, both HMB and EPA seemed to be equally effective, whereas the combination did seem to reduce p42 expression further. Expression of the ubiquitin-conjugating enzyme, $E2_{14k}$, was also reduced by both HMB and EPA, whereas the combination caused a further reduction in expression (Fig. 7). These results confirm HMB to be as effective as EPA in attenuating loss of muscle mass, protein degradation, and down-regulation of the ubiquitin-proteasome proteolytic pathway. This mechanism is likely to be at least partly responsible for the preservation of muscle mass in cachectic mice bearing the MAC16 tumor.

EPA has been shown to attenuate the increase in protein degradation in skeletal muscle of mice bearing the MAC16 tumor but has no effect on the depression of protein synthesis (23). In contrast, HMB, when evaluated at two dose levels (0.25 and 2.5 g/kg; Fig. 8*A*) not only attenuated protein degradation but also significantly increased protein synthesis in gastrocnemius muscle of mice bearing the MAC16 tumor when compared with control animals receiving PBS. This resulted in an increase in the ratio of protein synthesis to protein degradation in muscle (Fig. 8*B*) by 14-fold with HMB at 0.25 g/kg and 32-fold at 2.5 g/kg.

Discussion

Cancer-induced weight loss is an important contributor to death in cancer patients, and for many tumor types there is an inverse relationship between the degree of weight loss and the median survival time (1). Death normally occurs when the body weight loss reaches 30%, and such patients show an 85% reduction of body fat and 75% reduction is muscle protein (2). Despite the importance of cachexia, very few agents are able to counter the weight loss, especially the depletion of lean body mass. One of the few agents available is EPA, which preserves muscle mass by attenuating the increased protein degradation, but it has no effect on protein synthesis (23). EPA has been shown to produce an effect on protein

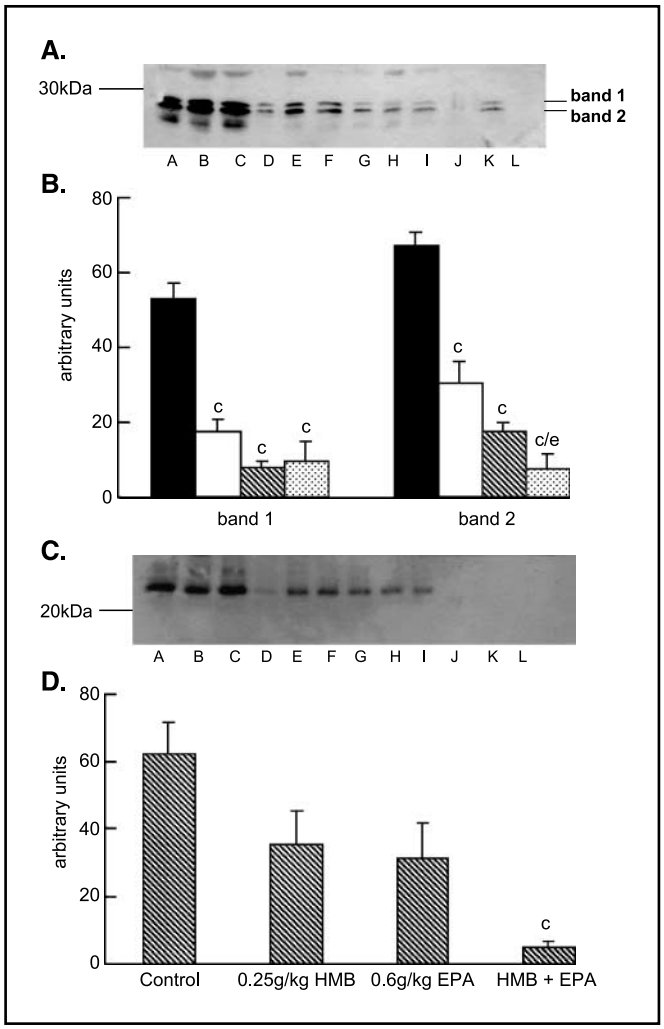

Figure 5. *A,* expression of proteasome 20S α subunits, detected by Western blotting, in gastrocnemius muscle of mice treated with PBS (*lanes A-C*), 0.25 g/kg HMB (*lanes D-F*), 0.6 g/kg EPA (*lanes G-I*), or the combination (*lanes J-L*). Treatment was terminated after 3 days. *B,* densitometric analysis of the blot shown in *A*. $n = 6$. Differences from control: c, $P < 0.001$; differences from HMB: e, $P < 0.01$. *C,* expression of proteasome 20S β subunits, detected by Western blotting, in gastrocnemius muscle of mice treated with PBS (*lanes A-C*), 0.25 g/kg HMB (*lanes D-F*), 0.6 g/kg EPA (*lanes G-I*), or the combination (*lanes J-L*). Treatment was terminated after 3 days. *D,* densitometric analysis of the blot shown in *C*. $n = 6$. Differences from control: c, $P < 0.001$.

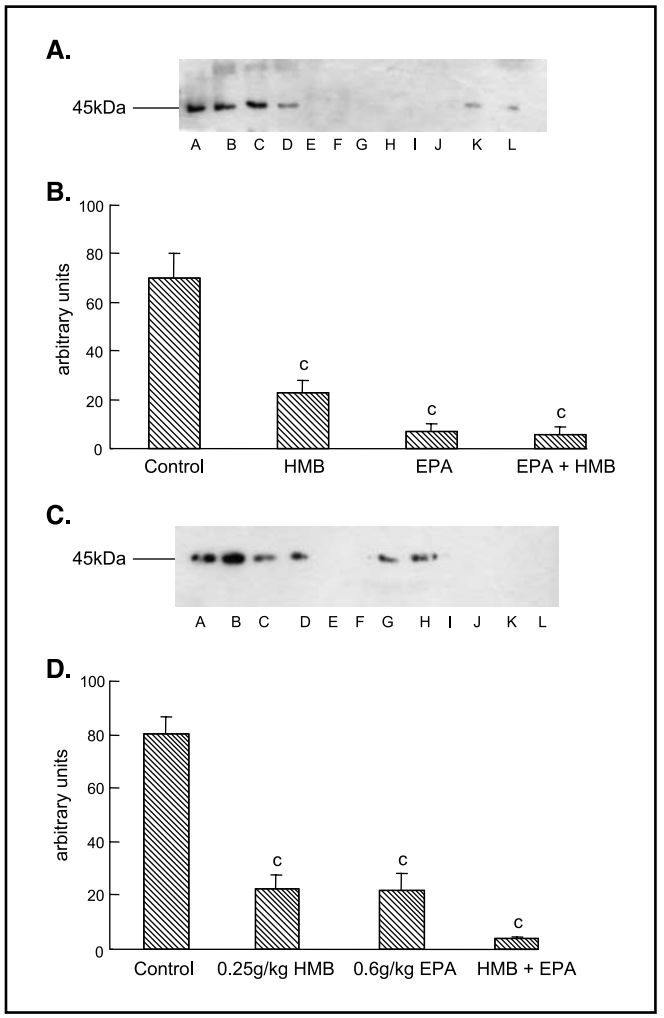

Figure 6. *A,* expression of proteasome 19S subunit, MSS1, detected by Western blotting, in gastrocnemius muscle of mice treated with PBS (*lanes A-C*), 0.25 g/kg HMB (*lanes D-F*), 0.6 g/kg EPA (*lanes G-I*), or the combination (*lanes J-L*). Treatment was terminated after 3 days. *B,* densitometric analysis of the blot shown in *A*. $n = 6$. Differences from control: c, $P < 0.001$. *C,* expression of proteasome 19S subunit, p42, detected by Western blotting, in gastrocnemius muscle of mice treated with PBS (*lanes A-C*), 0.25 g/kg HMB (*lanes D-F*), 0.6 g/kg EPA (*lanes G-I*), or the combination (*lanes J-L*). Treatment was terminated after 3 days. *D,* densitometric analysis of the blot shown in *C*. $n = 6$. Differences from control: c, $P < 0.001$.

degradation by inhibiting the action of a tumor catabolic factor, proteolysis-inducing factor, through suppression of eicosanoid production in muscle cells (24). This study shows that HMB is not only capable of attenuating protein degradation in skeletal muscle of cachectic mice but also stimulates protein synthesis resulting in an increase in the nonfat carcass mass. The mechanism of stimulation of protein synthesis has not been investigated, but because HMB is a metabolite of leucine it may follow a similar mechanism (25).

Clinical studies have shown the ability of HMB to increase lean body mass and muscle strength in humans undergoing progressive resistance-exercise training (26) and to increase body mass and fat-free mass in patients with cancer-induced weight loss (15). Similar body composition changes were seen in cachectic mice treated with HMB. The mechanism of this effect seems to arise from the ability of HMB to slow protein breakdown arising from muscle damage in exercise- (14) or in cancer-induced weight loss (15), although the action on protein breakdown has not been determined. There are three main proteolytic pathways: lysosomal, calcium-activated, and the ATP-ubiquitin-proteasome pathway. The lysosomal system seems to be involved in proteolysis of extracellular proteins and cell surface receptors and is not involved in the breakdown of myofibrillar proteins (27). However, the ubiquitin-proteasome system is considered to be the most important pathway for intracellular protein degradation in a range of catabolic conditions including starvation, sepsis, metabolic acidosis, weightlessness, severe trauma, denervation atrophy, and cancer-induced weight loss (28), although the calcium-calpain pathway has been suggested (29) to release myofilaments from the sarcomere in an early, perhaps rate-limiting, step in the process. In ubiquitin-dependent proteolysis proteins for degradation are recognized by a ubiquitin-ligase (E3), which also participates in the attachment of a polyubiquitin chain together with the ubiquitin-conjugating enzyme (E2). The E3 has been suggested to be rate limiting for ubiquitin conjugation (30). Protein degradation occurs within the proteasome, a cylindrical structure appearing as a stack of four rings, two outer α rings, and two inner β rings. Protein degradation occurs on three of the β subunits, whereas the α subunits are structural. The proteasome is capped with two terminal regulatory subunits (19S complex), which when combined with the proteasome core (20S) forms the 26S proteasome. ATPases associated with the 19S complex provide a supply of energy for unfolding proteins, so that they can penetrate the inner channel of the 26S proteasome. It has been suggested (31) that proteasome catalytic activity rather than substrate ubiquitination is the rate-limiting step of the overall pathway.

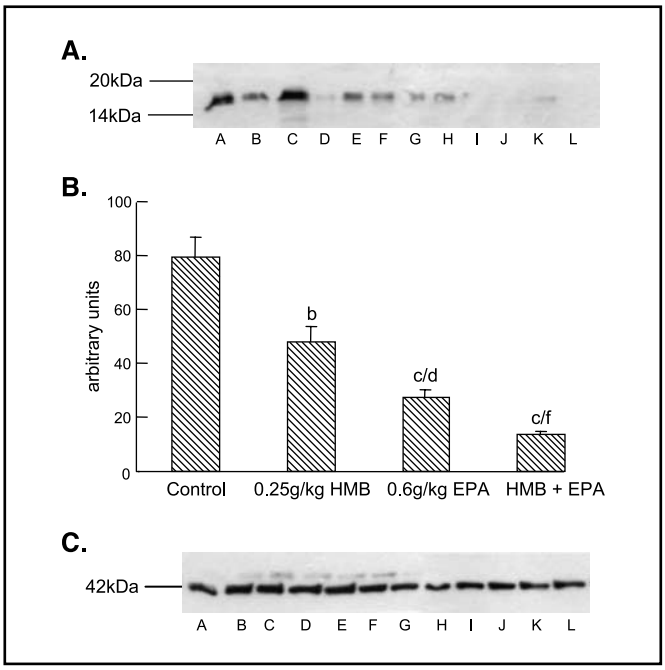

Figure 7. *A,* expression of $E2_{14k}$, detected by Western blotting, in gastrocnemius muscle of mice treated with PBS (*lanes A-C*), 0.25 g/kg HMB (*lanes D-F*), 0.6 g/kg EPA (*lanes G-I*), or the combination (*lanes J-L*). Treatment was terminated after 3 days. *B,* densitometric analysis of the blot shown in *A*. $n = 6$. Differences from control: b, $P < 0.01$; c, $P < 0.001$; differences from HMB alone: d, $P < 0.05$; f, $P < 0.001$. C. Actin loading control for the samples used in the blots shown in Figs. 5 to 7.

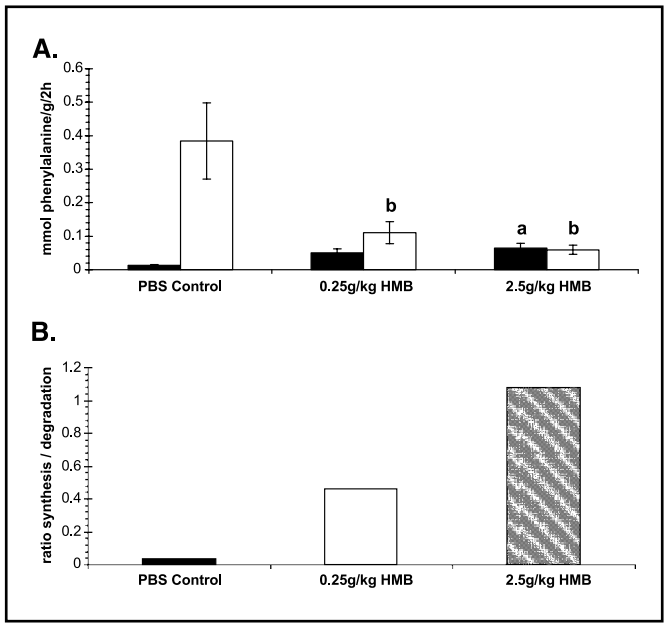

Figure 8. *A,* effect of daily p.o. administration of 2.5 g/kg HMB on the rate of protein synthesis (■) and degradation (□) in the gastrocnemius muscles of MAC16 tumor–bearing animals, expressed as millimoles of phenylalanine incorporated or released per gram of muscle per 2 hours. Treatment was terminated after 3 days. *Columns,* mean ($n = 6$); *bars,* SE. Differences from PBS control: a, $P < 0.05$; b, $P < 0.001$. *B,* ratio of the rate of protein synthesis to the rate of protein degradation in gastrocnemius muscles of mice treated in *A*.

This study has shown that HMB seems to be equivalent to EPA in attenuating the development of cachexia in mice bearing the MAC16 tumor. The equivalent human dose of HMB to that used in the mouse experiments would be 1.3 g/day, which is about half of that used in the effective treatment of weight loss in cancer patients (15). Both HMB and EPA produced a reduction in protein degradation in skeletal muscle by down-regulating the increased expression of the ubiquitin-proteasome pathway. Thus, both EPA and HMB reduced protein expression of the 20S proteasome α and β subunits, as well as two subunits of the 19S regulator MSS1 and p42, expression of $E2_{14k}$, and proteasome proteolytic activity. There was some evidence for a synergistic action between the two agents, but for most systems, maximal inhibition was seen with either agent alone. The mechanism by which HMB attenuated this system is the subject of a separate investigation (32), which shows that HMB, like EPA, interferes with intracellular signal transduction by proteolysis-inducing factor leading to increased proteasome expression.

This study provides a mechanistic interpretation for the ability of HMB to increase fat-free mass in patients with both cancer cachexia (15) and acquired immunodeficiency syndrome (33). Not only is HMB therapeutically effective but it is without any adverse effects on health in either healthy or diseased people (34). Indeed. the only effects on health noted were positive indicators, including an improved emotional profile, decreased feeling of weakness, and improvement in hematologic parameters. Thus, HMB may be safely used either alone or in combination with EPA to treat muscle atrophy in cancer cachexia and acquired immunodeficiency syndrome.

Acknowledgments

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