Am J Physiol Endocrinol Metab 293: E923–E931, 2007.
First published July 3, 2007; doi:10.1152/ajpendo.00314.2007.

Signaling pathways initiated by β-hydroxy-β-methylbutyrate to attenuate the depression of protein synthesis in skeletal muscle in response to cachectic stimuli

Helen L. Eley,[1] Steven T. Russell,[1] Jeffrey H. Baxter,[2] Pradip Mukerji,[2] and Michael J. Tisdale[1]

[1]*Nutritional Biomedicine, School of Life and Health Sciences, Aston University, Birmingham B4 7ET, United Kingdom; and* [2]*Ross Products Division, Abbott Laboratories, Columbus, Ohio*

Submitted 29 May 2007; accepted in final form 2 July 2007

Eley HL, Russell ST, Baxter JH, Mukerji P, Tisdale MJ. Signaling pathways initiated by β-hydroxy-β-methylbutyrate to attenuate the depression of protein synthesis in skeletal muscle in response to cachectic stimuli. *Am J Physiol Endocrinol Metab* 293: E923–E931, 2007. First published July 3, 2007; doi:10.1152/ajpendo.00314.2007.—To investigate the mechanism by which β-hydroxy-β-methylbutyrate (HMB) attenuates the depression of protein synthesis in the skeletal muscle of cachectic mice, a study has been carried out in murine myotubes in the presence of proteolysis-inducing factor (PIF). PIF inhibited protein synthesis by 50% within 4 h, and this was effectively attenuated by HMB (25–50 μM). HMB (50 μM) alone stimulated protein synthesis, and this was attenuated by rapamycin (27 nM), an inhibitor of mammalian target of rapamycin (mTOR). Further evidence for an involvement of this pathway was shown by an increased phosphorylation of mTOR, the 70-kDa ribosomal S6 kinase ($p70^{S6k}$), and initiation factor 4E-binding protein (4E-BP1) and an increased association of eukaryotic initiation factor 2 (eIF4E) with eIF4G. PIF alone induced a transient (1–2 h) stimulation of phosphorylation of mTOR and $p70^{S6k}$. However, in the presence of HMB, phosphorylation of mTOR, $p70^{S6k}$, and 4E-BP1 was increased, and inactive 4E-BP1-eIF4E complex was reduced, whereas the active eIF4G·eIF4E complex was increased, suggesting continual stimulation of protein synthesis. HMB alone reduced phosphorylation of elongation factor 2, but this effect was not seen in the presence of PIF. PIF induced autophosphorylation of the double-strand RNA-dependent protein kinase (PKR), leading to phosphorylation of eIF2 on the α-subunit, which would inhibit protein synthesis. However, in the presence of HMB, phosphorylation of PKR and eIF2α was attenuated, and this was also observed in skeletal muscle of cachectic mice administered HMB (0.25 g/kg). These results suggest that HMB attenuates the depression of protein synthesis by PIF in myotubes through multiple mechanisms.

proteolysis-inducing factor; eukaryotic initiation factor 2α; 4E-binding protein 1

MUSCLE PROTEINS UNDERGO constant change because of remodeling, with synthesis of new proteins and breakdown of old or damaged proteins. Normally these two processes are in check so that muscle mass remains relatively constant. However, in certain catabolic conditions, such as cancer cachexia, protein breakdown exceeds synthesis of new proteins, and muscle atrophy occurs (11). The increase in protein degradation appears to be the result of an increased expression of the ubiquitin-proteasome proteolytic pathway (18). The mechanism for the depression in protein synthesis is unknown, although it appears not to be caused by either insufficient insulin or amino acids (20, 21). Changes in muscle protein homeostasis are probably induced by cytokines, such as tumor necrosis factor-α, or tumor factors such as proteolysis-inducing factor (PIF), which inhibit muscle protein synthesis and stimulate degradation (38).

Loss of skeletal muscle can be attenuated by agents such as eicosapentaenoic acid (EPA), which acts to suppress protein catabolism by inhibition of the increased activity and expression of the ubiquitin-proteasome proteolytic pathway in the muscle of cachectic animals (41). However, EPA has no effect on the depression of protein synthesis, so that muscle bulk cannot increase (32). In contrast, the amino acids leucine, arginine, and methionine are capable of doubling the rate of protein synthesis (32), and when combined with EPA produce a significant increase in the ratio of protein synthesis to degradation.

Loss of skeletal muscle can also be attenuated by agents such as β-hydroxy-β-methylbutyrate (HMB) a metabolite of leucine that both inhibits protein degradation and stimulates protein synthesis in the muscles of cachectic mice (34). HMB (3 g/day), combined with L-arginine and L-glutamine (both at 14 g/day), has undergone clinical evaluation in patients with cancer cachexia and has been shown to stimulate an increase in body weight through an increase in lean body mass (22).

The effect of HMB on protein degradation has been shown to be the result of inhibition of the PIF-induced increase in the ubiquitin-proteasome pathway, through attenuation of cellular signaling pathways leading to activation of the transcription factor nuclear factor-κB (33), which has been shown to cause severe muscle wasting in mice (8). However, the mechanism of the stimulation of protein synthesis is not known. Branched-chain amino acids, such as leucine, have been shown to regulate translation initiation of protein synthesis by acting as modulators of intracellular signal transduction pathways. Thus muscle protein synthesis is stimulated through activation of the mRNA-binding step in translation initiation. This occurs through binding of the 5′-cap structure recognition [containing 7-methylguanosine triphosphate (m^7GTP)] of the mRNA to initiation factor 4E (eIF4E), a subunit of the initiation complex eIF4F (13). The availability of eIF4E for complex formation is regulated by reversible association with its binding protein 4E-BP. Hypophosphorylated 4E-BP1 blocks eIF4F assembly because it competes with eIF4G for binding to eIF4E. Leucine

Address for reprint requests and other correspondence: M. J. Tisdale, Nutritional Biomedicine, School of Life and Health Sciences, Aston Univ., Birmingham B4 7ET, UK (e-mail: m.j.tisdale@aston.ac.uk).

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induces hyperphosphorylation of 4E-BP1, resulting in the release of eIF4E from the inactive 4E-BP1-eIF4E complex, allowing it to associate with eIF4G to form the active eIF4F complex (2). Leucine also stimulates the phosphorylation and activation of the 70-kDa ribosomal S6 kinase ($p70^{S6k}$), which confers selective translation of mRNAs that contain a polypyrimidine tract and which are involved in the formation of the translation apparatus and elongation factors (1). Rapamycin inhibited both of these processes, suggesting that it occurred through the mammalian target of rapamycin (mTOR) pathway. Leucine has been shown not to cause significant alterations in either the activity of eukaryotic initiation factor (eIF) 2B or the phosphorylation of eIF2α (2). Because HMB is a metabolite of leucine, this suggests that it may have a similar effect on protein synthesis, and it is hypothesized that it will attenuate the depression of protein synthesis through hyperphosphorylation of 4E-BP1 and phosphorylation of mTOR and $p70^{S6k}$.

The current study examines the mechanism of the stimulatory effect of HMB on protein synthesis, both in vivo in mice bearing the cachexia-inducing MAC16 tumor and in vitro in myotubes exposed to PIF.

MATERIALS AND METHODS

Materials. FCS, horse serum (HS), DMEM, and antibodies were purchased from Life Technologies (Paisley, Scotland). HMB (as the calcium salt) was obtained from Organic Technologies (Coshocton, OH). L-[2 6-^{3}H]phenylalanine (sp act 2.00 TBq/mmol), m^7GTP Sepharose 4B, Hybond A nitrocellulose membranes, and enhanced chemiluminescence (ECL) development kits were from Amersham Pharmacia Biotech (Bucks, UK). Rabbit polyclonal antisera to 4E-BP1, eIF4E, phosphor-eIF4E (Ser^{209}), eIF4G, phosphor-eIF4G (Ser^{1108}), mTOR, phosphor-$p70^{S6k}$ (Thr^{389}) , total $p70^{S6k}$, phosphor-elongation factor 2 (eEF2; Thr^{56}), and rabbit monoclonal antibodies to phosphor-4E-BP1 ($Thr^{37/46}$), phosphor- and total double-strand RNA-dependent protein kinase (PKR), and phosphor-mTOR (Ser^{2448}) were purchased from New England Biolabs (Herts, UK). Rabbit polyclonal antisera to phosphor-eIF2α was from Abcam (Cambridge, UK) and to total eIF2α from Santa Cruz Biotechnology (Santa Cruz, CA). Peroxidase-conjugated goat anti-rabbit antibody was purchase from Dako (Cambridge, UK). PhosphosafeExtraction Reagent, Salubrinal, and the PKR inhibitor were from Merck Eurolab (Leics, UK). The C_2C_{12} myoblasts were obtained from the European Collection of Cell Cultures (Salisbury, UK).

Animals. Pure strain male NMRI mice (minimum weight 25 g), bred in our own colony, were transplanted subcutaneously in the flank with fragments of the MAC16 tumor, obtained from donor animals with established weight loss. The procedure for tumor transplantation has been described previously (5). When weight loss was ~5%, usually 12–15 days after tumor transplantation, animals were randomized into groups of six to receive solvent (PBS) or HMB (0.25 g/kg) administered per os by gavage daily as described (34). Tumor volume, body weight, and food and water intake were measured daily. When the body weight loss reached 20% of the maximal body weight after tumor transplantation, the animals were terminated by cervical dislocation, and soleus muscle was removed for further analysis by Western blotting. Non-tumor-bearing animals of the same sex, age, and weight as the tumor-bearing animals at transplantation served as a control. The results of this study are shown in Fig. 6. Protein synthesis in soleus muscle was determined by the incorporation of L-[2,6-^{3}H] phenylalanine, as described (34). When the animals were terminated, the soleus muscles were quickly dissected out and maintained in isotonic ice-cold saline. Protein synthesis was determined over a 2-h period at 37°C, in which the isolated soleus muscles were incubated in RPMI 1640, without phenol red, saturated with O_2-CO_2 (19:1). After incubation, muscles were rinsed in nonradioactive medium, blotted, and homogenized in 4 ml of 2% perchloric acid. The rate of protein synthesis was calculated by dividing the amount of protein-bound radioactivity by the amount of acid-soluble radioactivity. Further details on animal treatments is also given in the legend to Fig. 1. All animal experiments followed a strict protocol, approved by the British Home Office, and the ethical guidelines that were followed met the standards required by the UK Co-ordinating Committee on Cancer Research (42).

Myogenic cell culture. C_2C_{12} myoblasts were passaged in DMEM supplemented with 10% FCS, 1% glutamine, and 1% penicillin-streptomycin under an atmosphere of 10% CO_2 in air at 37°C. When the myoblasts reached confluency, they were allowed to differentiate into myotubes by replacement of the propagation medium with DMEM containing 2% HS with medium changes every 2 days. Differentiation was complete in 5–7 days.

Purification of PIF. PIF was purified from solid MAC16 tumors excised from mice with a weight loss between 20 and 25%, as previously described (39). Tumors were homogenized in 10 mM Tris·HCl, pH 8.0, containing 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM EGTA, and 1mM dithiothreitol and precipitated with solid ammonium sulfate (40% wt/vol). The supernatant was subjected to affinity chromatography using anti-PIF monoclonal antibody coupled to a solid matrix. The immunogenic fractions were concentrated and used for further studies.

Measurement of protein synthesis. Myotubes were formed in six-well multiwell dishes and were supplemented with DMEM without HS and phenol red 18 h before experimentation. PIF was added to the cultures at a concentration of 4.2 nM, which has been shown previously (10) to cause the maximum depression of protein synthesis. HMB was added to the cultures 2 h before PIF and at concentrations (25 and 50 μM) previously found effective in the attenuation of protein degradation induced by PIF (33). Rapamycin (27 nM), an inhibitor of mTOR (1), Salubrinal (15 μM), a selective inhibitor of eIF2α dephosphorylation (7), and the PKR inhibitor 8-[1-(1H-imidazol-4-yl)meth-(z)-ylidene]-6,8-dihydrothiazol[5,4-e]indol-7-one (210 nM; see Ref. 16) were also added 2 h before PIF. To determine protein synthesis, cultures were supplemented with 2 μl (370 kBq) L-[2, 6-^{3}H] phenylalanine (sp act 196 TBq/mmol) in 8 μl sterile PBS, and the plates were incubated for 4 h at 37°C under an atmosphere of 10% CO_2 in air. Protein synthesis was linear over a 24-h period. The reaction was arrested by washing three times with 1 ml ice-cold sterile PBS. Following removal of PBS, 1 ml ice-cold 0.2 M perchloric acid was added, and the plates were kept at 4°C for 20 min. The perchloric acid was substituted with 1 ml of 0.3 M NaOH/well, and incubation was continued for 30 min at 4°C, followed by a further incubation at 37°C for 20 min. The NaOH extract was removed and combined with a further 1-ml wash of each well, and 0.5 ml of 0.2 M perchloric acid was added and left on ice for 20 min. The extract was then centrifuged at 700 *g* for 5 min at 4°C, the protein-containing pellet was dissolved in 1 ml of 0.3 M NaOH, and 0.5 ml of the solution was counted for radioactivity after mixing with 8 ml Ultima Gold XR scintillation fluid. To measure the intracellular amino acid pool, the perchloric acid extract was neutralized with 0.2 M potassium hydroxide, and the insoluble potassium perchlorate was removed by centrifugation (4,500 *g*, 10 min). The radioactivity of the supernatant was determined as above.

Measurement of RNA. RNA was estimated in the perchloric acid supernatant in the protein synthesis experiment, by centrifugation of the samples at 700 *g* for 5 min at 4°C, and by measuring the absorbance at 232 and 260 nm. The following equation uses the modified formula of Ashford and Pain (3) and corrects for any absorbance because of the presence of aromatic amino acids.

$$\text{RNA (}\mu\text{g/ml)} = (A_{260} \times 32.9) - (A_{232} \times 8.11)$$

where A_{260} and A_{232} are absorbance at 260 and 232 nm, respectively.

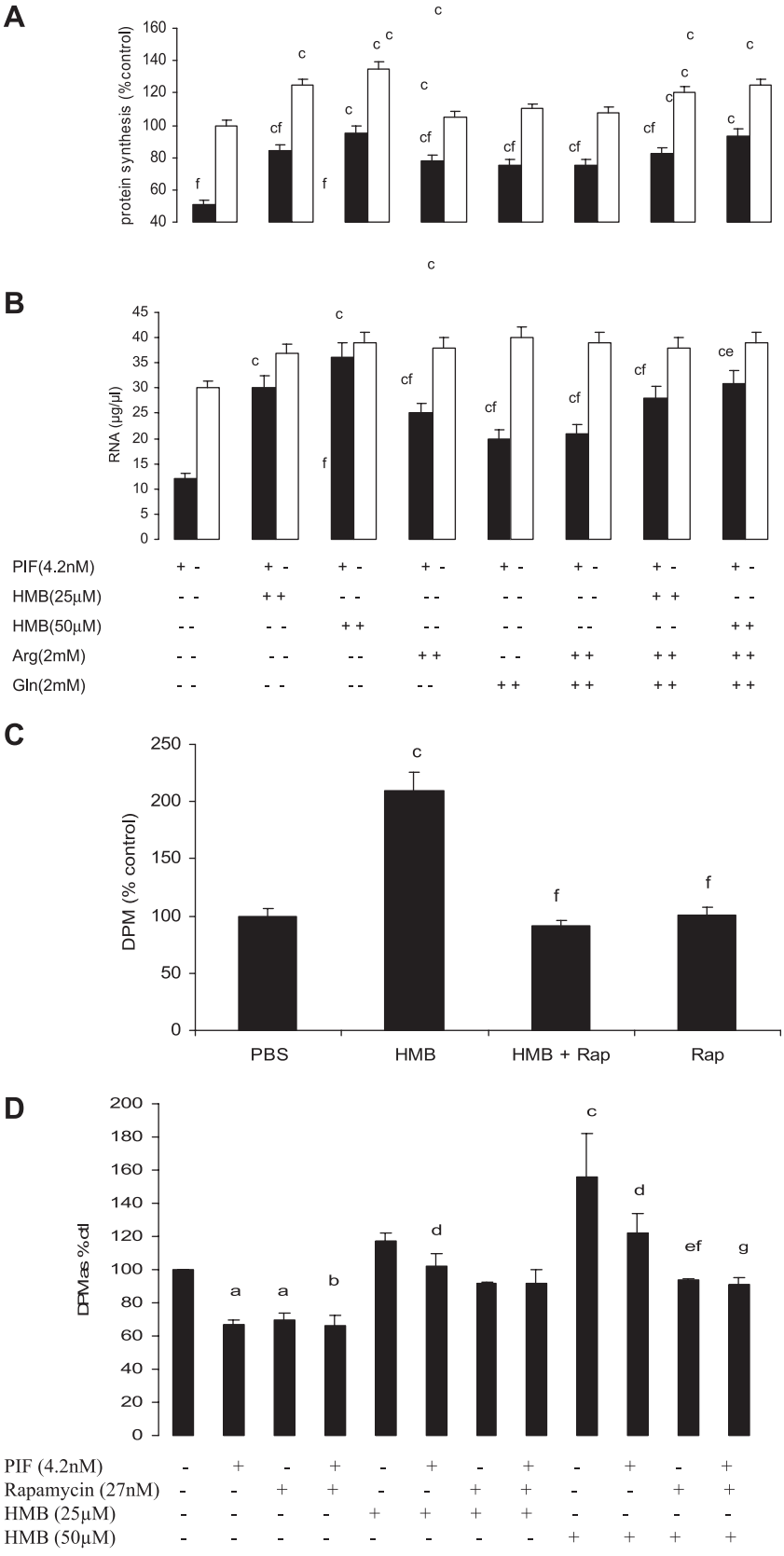

Fig. 1. *A*: effect of proteolysis-inducing factor (PIF, 4.2 nM) on protein synthesis in murine myotubes either alone, in the presence of β-hydroxy-β-methylbutyrate (HMB, 25 or 50 μM), L-arginine (Arg), or L-glutamine (Gln) (both at 2 mM), or with the combinations shown, added 2 h before PIF. *B*: RNA concentrations in murine myotubes 4 h after treatment with PIF or the combinations shown. Differences from control values are represented as $P < 0.001$ (c), whereas differences from untreated cultures in the presence of PIF are shown as $P < 0.05$ (e) or $P < 0.001$ (f). *C*: protein synthesis in soleus muscle of mice bearing the MAC16 tumor treated with HMB, rapamycin (Rap), or a combination, as described in MATERIALS AND METHODS. Animals bearing the MAC16 tumor were allowed to undergo a 10% loss of body weight and were then randomized into four groups ($n = 6$). The groups received the following treatments: *1*) control received PBS iv and 2 h later PBS oral and were terminated 90 min later; *2*) PBS iv and 2 h later CaHMB (0.25 g/kg) and terminated after a further 90 min; *3*) rapamycin (0.75 mg/kg) iv followed 2 h later by CaHMB (0.25 g/kg) and terminated 90 min later; and *4*) rapamycin (0.75 mg/kg) iv followed 2 h later by PBS and terminated 90 min later. DPM, disintegrations/min. Differences from control are shown as $P < 0.001$ (c), whereas differences from HMB are shown as $P < 0.001$ (f). *D*: effect of PIF (4.2 nM) on protein synthesis in murine myotubes in the absence and presence of HMB (50 μM), with or without rapamycin (27 nM). Ctl, Control. Differences from control cultures are shown as $P < 0.05$ (a), $P < 0.01$ (b), or $P < 0.001$ (c) and from PIF alone as $P < 0.001$ (d). Differences from rapamycin alone are shown as $P < 0.001$ (e), from HMB alone as $P < 0.001$ (f), and form HMB + PIF as $P < 0.001$ (g).

Western blot analysis. Myotubes were scraped off the plates and washed with PBS, followed by lysis in PhosphosafeExtraction Reagent by incubation for 5 min at room temperature. The lysate was cleared by centrifugation at 15,000 *g* for 10 min at 4°C, and samples of cytosolic protein were resolved on 10% SDS-PAGE and transferred to 0.45-μm nitrocellulose membranes, which had been blocked with 5% Marvel in PBS, pH 7.5, at 4°C overnight. The primary antibodies for PKR, mTOR, and $p70^{S6k}$ were used at a dilution of 1:1,000, except for eIF2α (1:500) and actin (1:200). The secondary antibodies were used at a dilution of 1:1,000. Incubation was for 1 h at room temperature (actin) or overnight, and development was by ECL. Blots were scanned by a densitometer to quantify differences.

m^7GTP-Sepharose chromatography. The association of 4E-BP1 or eIF4G with eIF4E was determined by quantitation of recovery when eIF4E was extracted with the affinity chromatography resin m^7GTP-Sepharose 4B. Myotubes were harvested and lysed in 150 mM NaCl, 1% Nonidet P-40, 50 mM Tris·HCl, pH 7.4, 0.25% sodium deoxycholate, 2 mM EGTA, 1 mM EDTA, 0.2 mM sodium orthovanadate, 20 mM NaF, and 1% protease inhibitor mixture at 4°C and left for a further 10 min at room temperature with occasional vortexing. The homogenate was centrifuged for 15 min at 15,000 *g*, and the supernatant was used for Western blotting of eEF2. Next the supernatant (250 μg protein/sample) was added to a microcentrifuge tube containing 80 μl of m^7GTP-Sepharose 4B at 4°C, and incubation was continued for 1 h. After low-speed centrifugation (13,000 *g*), and three washes with 1 ml lysis buffer, the slurry was resuspended in 80 μl 2× SDS sample buffer and subjected to electrophoresis on either 10% (eIF4E and eIF4G) or 15% (4E-BP1) SDS-PAGE, and the levels of eIF4E, eIF4G, and 4E-BP1 were quantified by Western blotting as described above. The amount of eIF4G and 4E-BP1 found in the fraction bound to m^7GTP-Sepharose represents their association with eIF4E.

Statistical analysis. Results are shown as means ± SE for at least three replicate experiments. Differences in means between groups were determined by one-way ANOVA followed by the Tukey-Kramer multiple-comparison test. *P* values <0.05 were considered significant.

RESULTS

PIF inhibited protein synthesis in murine myotubes by 50% within 4 h of addition, and this was effectively attenuated by HMB at a concentration of both 25 and 50 μM (Fig. 1*A*). There was no effect of combining HMB with the amino acids L-arginine and L-glutamine, as employed clinically, although L-arginine and L-glutamine alone did partially attenuate the depression of protein synthesis by PIF. There was no effect of the amino acids, or PIF, on the uptake of L-[2,6-^{3}H]phenylalanine in myotubes. PIF produced a significant reduction in the RNA content of the myotubes (Fig. 1*B*), and this was also effectively attenuated by HMB, and to a lesser extent L-arginine and L-glutamine. At a concentration of 50 μM, HMB alone stimulated protein synthesis in myotubes, and this was signif-

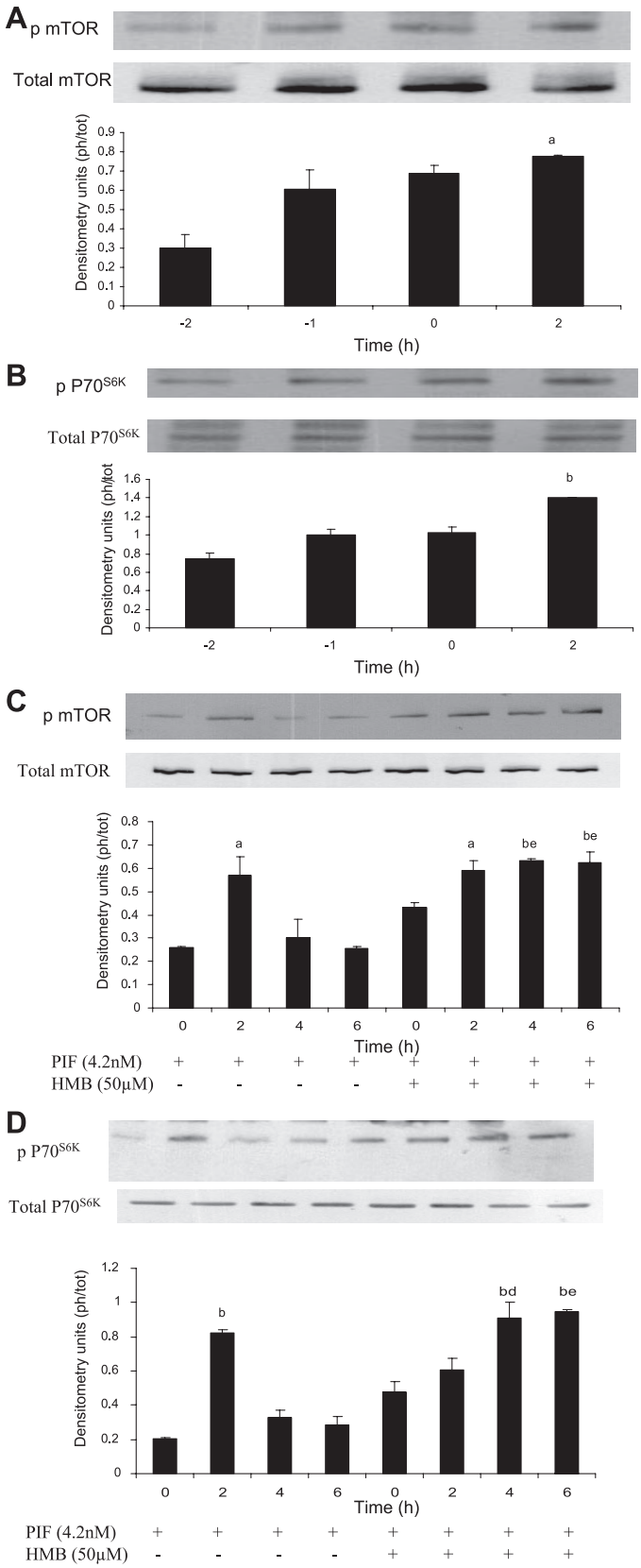

Fig. 2. Western blots for the effect of HMB (50 μM) alone on phosphorylation (p) of mammalian target of rapamycin (mTOR) on Ser^{2448} (*A*) and 70-kDa ribosomal S6 kinase ($p70^{S6k}$) at Thr^{389} (*B*) in murine myotubes. The HMB was added 2 h before the zero time point to correspond with the times in *C* and *D;* therefore, the first two lanes are shown as −2 and −1 h. The effect of PIF (4.2 nM) alone or in combination with HMB (50 μM), added 2 h before PIF, on phosphorylation (ph) of mTOR and $p70^{S6k}$ is shown in *C* and *D*, respectively. Total (tot) mTOR and $p70^{S6k}$ were used as loading controls, and the densitometric analysis represents the ratio of phosphorylated to total forms of mTOR and $p70^{S6k}$ and are an average of three separate Western blots. HMB was added 2 h before the PIF. Differences from control are shown as $P < 0.05$ (a) or $P < 0.01$ (b), whereas differences in the presence of HMB are shown as $P < 0.01$ (e).

icantly inhibited by rapamycin (27 nM), an inhibitor of mTOR, that phosphorylates and activates several components of the translational machinery, including the 70-kDa protein kinase that phosphorylates ribosomal protein S6 ($p70^{S6k}$; see Ref. 4 and Fig. 1*C*). This suggests that HMB may stimulate protein synthesis through the mTOR/$p70^{S6k}$ pathway. Rapamycin alone depressed protein synthesis to the same extent as PIF, but there was no further depression with the combination. Although rapamycin depressed protein synthesis in the presence of HMB, there was no further depression in the presence of PIF. A similar result was observed in vivo in soleus muscle of mice bearing the MAC16 tumor (Fig. 1*D*). HMB caused a twofold stimulation of protein synthesis that was completely attenuated by rapamycin. Rapamycin alone had no effect on protein synthesis in this model. This suggests that HMB alone may stimulate protein synthesis through the mTOR/$p70^{S6k}$ pathway and that this may be at least partly responsible for the attenuation of PIF inhibition of protein synthesis.

As with leucine (24) HMB stimulated phosphorylation and activation of both mTOR and $p70^{S6k}$ within 6 h of treatment (Fig. 2, *A* and *B*). Phosphorylation of mTOR at Ser^{2448} has been shown to have an important role in the control of protein synthesis (28). This confirms the earlier conclusion (Fig. 1) that HMB may stimulate protein synthesis through the mTOR/$p70^{S6k}$ pathway. PIF alone caused transient stimulation of phosphorylation of both mTOR and $p70^{S6k}$ within 2 h of treatment, but this returned to baseline values within 4 h (Fig. 2, *C* and *D*). Short-term activation of mTOR and $p70^{S6k}$ has been observed previously in myotubes in the presence of PIF. This probably represents the need for protein synthesis for formation of more proteasome subunits and ubiquitin ligases, which are required for protein degradation to occur (14) in the presence of attenuated protein synthesis. In the presence of HMB, phosphorylation of both mTOR and $p70^{S6k}$ was evident within 2 h of PIF treatment (Fig. 2, *C* and *D*) and was maintained for the 6 h of observation. This might be expected to lead to a continuing stimulation of protein synthesis.

The translational repressor 4E-BP1 is regulated through mTOR and undergoes phosphorylation at multiple sites, which affects its function (26). During SDS-PAGE, 4E-BP1 was resolved into multiple electrophoretic forms (α, β, and γ), representing the differentially phosphorylated forms, with the γ-form having the slowest electrophoretic mobility (Fig. 3*A*). PIF alone had no effect on phosphorylation of 4E-BP1, but HMB alone and in combination with PIF produced a fourfold increase in 4E-BP1 phosphorylation (Fig. 3*A*) and a 50% decrease in the amount of 4E-BP1 associated with eIF4E (Fig. 3*B*). In addition, phosphorylation of eIF4G in the eIF4G·eIF4E complex was increased twofold (Fig. 3*C*), and the total eIF4G in the complex was also increased (Fig. 3*D*). There was no significant effect of any of the treatments on eIF4E phosphorylation (data not shown), although there was a tendency for a decrease with HMB that did not reach significance. Leucine has previously been shown (1) to inhibit phosphorylation of eIF4E.

Previous results (10) have shown that PIF inhibits protein synthesis in myotubes by activation of PKR, through autophosphorylation, resulting in an increased phosphorylation of eIF2 on the α-subunit. As shown in Fig. 4*A*, inhibition of PKR by a low-molecular-weight inhibitor (16) attenuated the depression in protein synthesis produced by PIF. Although the stimulation

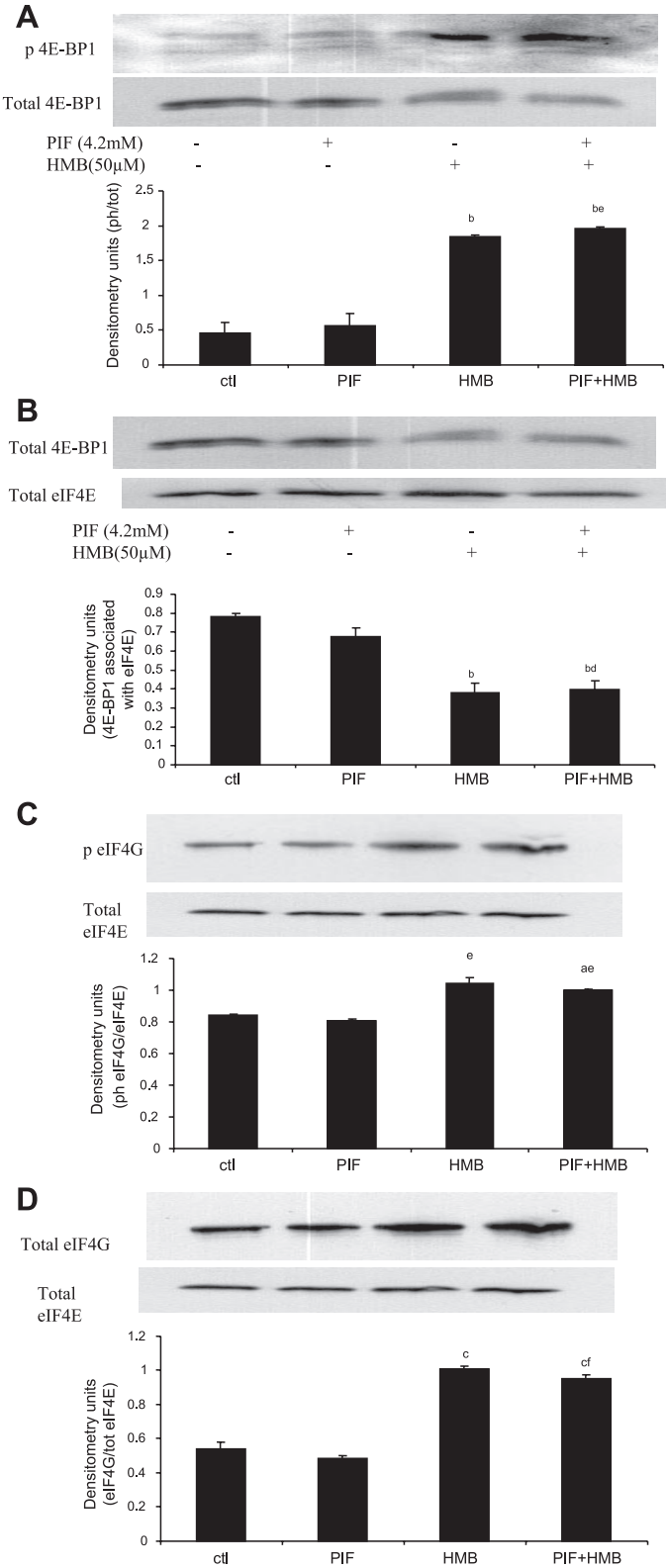

Fig. 3. *A*: phosphorylation of eukaryotic initiation factor (eIF) 4E-binding protein (4E-BP1) at $Thr^{37/46}$ in myotubes after 4 h treatment with PIF (4.2 nM) with or without HMB (50 μM) and histogram showing the amount of 4E-BP1 in the γ-phosphorylated form as a percentage of the total 4E-BP1. *B*: amount of 4E-BP1 associated with eIF4E. *C*: amount of phosphorylated eIF4G at Ser^{1108} associated with eIF4E. *D*: amount of eIF4G associated with eIF4E. Differences from control are shown as $P < 0.01$ (b) or $P < 0.01$ (c), whereas differences from PIF alone are indicated as $P < 0.05$ (a), $P < 0.01$ (e), or $P < 0.001$ (f).

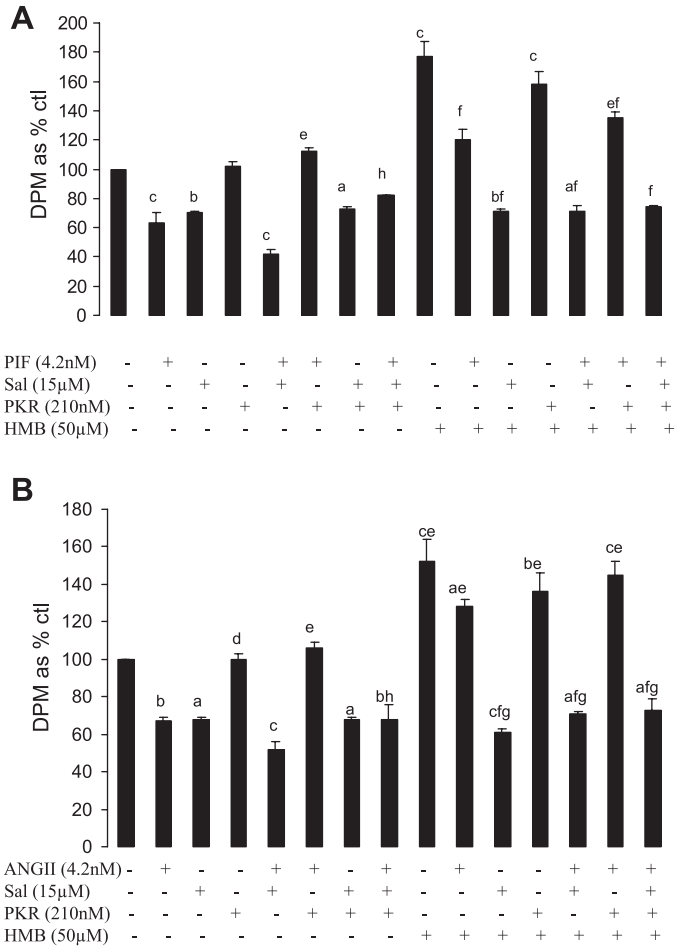

Fig. 4. Effect of HMB (50 μM), double-strand RNA-dependent protein kinase (PKR) inhibitor (210 nM), and Salubrinal (Sal, 15 μM) on protein synthesis in murine myotubes in the presence of PIF (*A*) or ANG II (*B*). Differences from control are shown as $P < 0.05$ (a), $P < 0.01$ (b), or $P < 0.001$ (c). Differences from PIF or ANG II alone are shown as $P < 0.01$ (d) or $P < 0.001$ (e). Differences from HMB are shown as $P < 0.001$ (f) and from HMB + PIF (or ANG II) as $P < 0.001$ (g). Difference from PIF or ANG II plus PKR inhibitor, $P < 0.001$ (h).

of protein synthesis by HMB alone was not affected by the PKR inhibitor, treatment with Salubrinal, a selective inhibitor of eIF2α dephosphorylation, (7), significantly decreased protein synthesis in the presence of HMB, suggesting that at least part of its effect is mediated through inhibition of PKR (Fig. 4*A*). This suggestion is supported by the ability of Salubrinal to attenuate the stimulation of protein synthesis by HMB in the presence of PIF. A similar result was obtained with ANG II (Fig. 4*B*), which has also been shown to attenuate protein synthesis in myotubes through an increased phosphorylation of eIF2α (10). HMB also attenuated the depression of protein synthesis induced by ANG II, and, as with PIF, this was reversed in the presence of Salubrinal (Fig. 4*B*).

To determine the effect of HMB on activation (autophosphorylation) of PKR, and subsequent phosphorylation of eIF2α, myotubes were treated with PIF over a 6-h time period, and levels of phosphor-PKR and eIF2α compared with total PKR and eIF2α were determined at time intervals (Fig. 5). As previously reported (10), PIF induced an increased phosphorylation of both PKR and eIF2α, which became significant

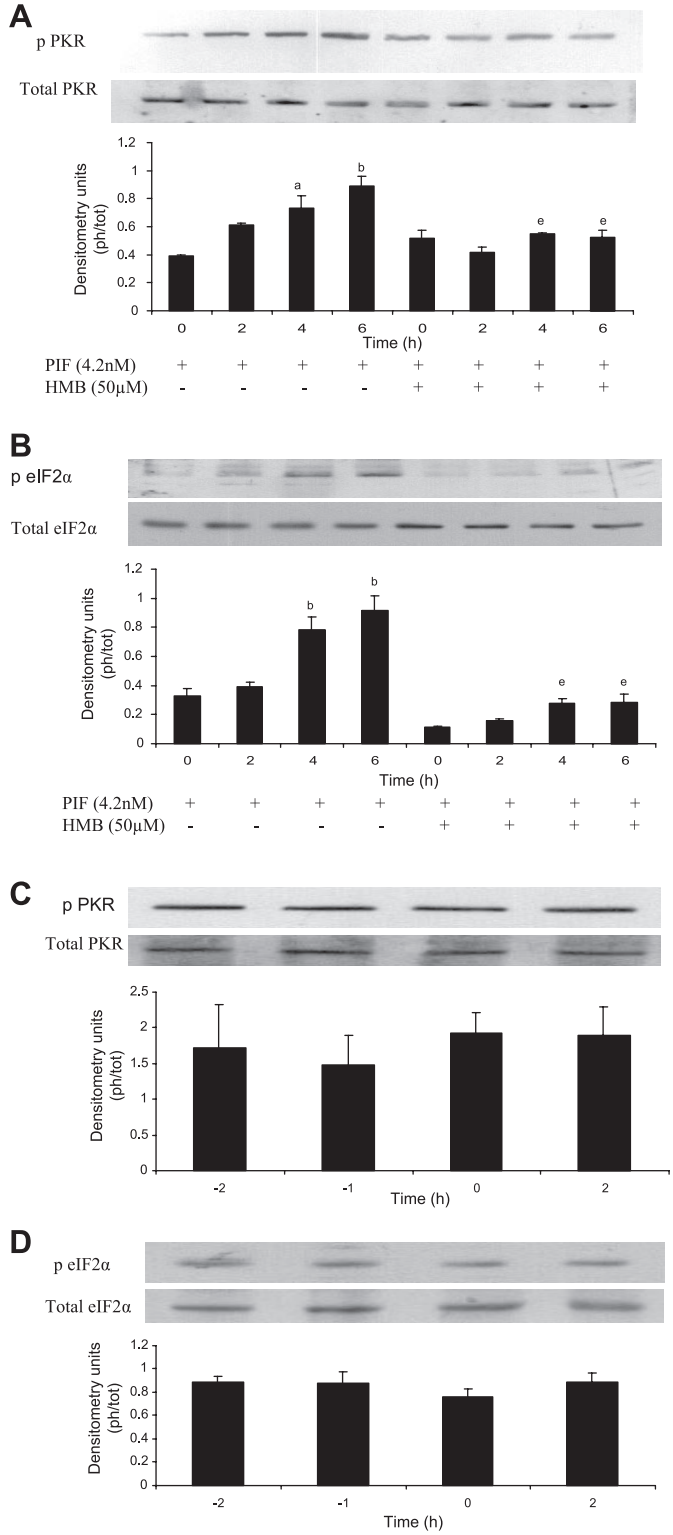

Fig. 5. Effect of PIF (4.2 nM) on phosphorylation of PKR (*A*) and eIF2α (*B*) in murine myotubes over a 6-h period in the absence and presence of HMB (50 μM), as determined by Western blotting. The HMB was added 2 h before PIF. The densitometric analysis is the average of 3 separate Western blots and represents the ratio of the phosphorylated to total forms of PKR and eIF2α. Differences from control are indicated as $P < 0.05$ (a) or $P < 0.01$ (b), whereas differences in the presence of HMB are indicated as $P < 0.01$ (e). The effect of HMB (50 μM) alone on phosphorylation of PKR and eIF2α is shown in *C* and *D*, respectively. The HMB was added 2 h before the zero time point to correspond with the times in *A* and *B*; therefore, the first two lanes are shown as −2 and −1.

within 2 and 4 h, respectively (Fig. 5, *A* and *B*). HMB alone had no effect of the phosphorylation of either PKR or eIF2α (Fig. 5, *C* and *D*). However, HMB completely attenuated autophosphorylation of PKR in the presence of PIF (Fig. 5*A*), as well as the ability of PIF to induce phosphorylation of eIF2α (Fig. 5*B*). These results suggest that HMB inhibits phosphorylation of eIF2α in the presence of PIF by preventing activation of PKR.

To determine whether the same effect may be occurring, in vivo muscles from mice bearing the MAC16 tumor, which has been administered HMB (0.25 kg^{-1}) and which led to attenuation of weight loss (Fig. 6*A*), were Western blotted for expression of phosphor-PKR and eIF2α (Fig. 6, *B* and *C*). Compared with non-tumor-bearing controls, muscle from mice bearing the MAC16 tumor showed an increased level of phosphorylation of both PKR and eIF2α, and this was completely attenuated in animals administered HMB, down to levels found in non-tumor-bearing animals.

The phosphorylation of eEF2 was investigated as an index of the capacity of translation elongation. With the use of an antibody that detects eEF2 only when it is phosphorylated at its main site of phosphorylation, Thr^{56} (23), PIF was shown to produce a small nonsignificant increase in phosphor-eEF2 levels, whereas HMB produced a decrease compared with control (Fig. 6*D*). However, the combination of HMB with PIF showed significantly higher levels of phosphor-eEF2 than HMB alone.

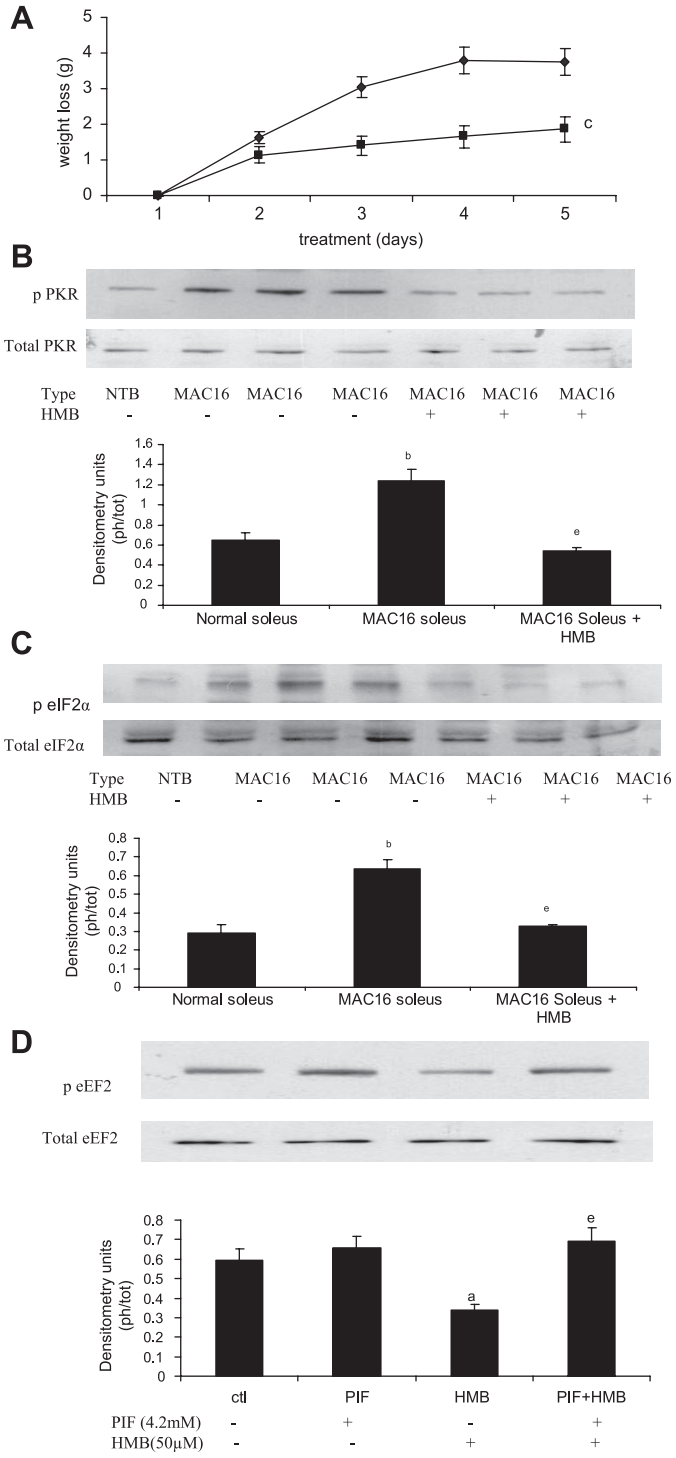

Fig. 6. *A*: weight changes in NMRI mice bearing the MAC16 tumor treated daily with either PBS (◆) or HMB (0.25 g/kg; ■), as described in MATERIALS AND METHODS. Differences from PBS are indicated as $P < 0.001$ (c). *B*: Western blot for the effect of HMB on phosphorylation of PKR and eIF2α (*C*) in soleus muscle of mice bearing the MAC16 tumor (MAC16) and in non-tumor-bearing mice (NTB). Each lane represents the soleus muscle from an individual mouse, and the densitometric analysis is the ratio of the phospho to total forms. Differences from control (NTB) are shown as $P < 0.01$ (b), whereas differences from tumor-bearing mice administered PBS are shown as $P < 0.01$ (e). *D*: Western blot of the effect of PIF (4.2 nM), HMB (50 μM), or the combination on phosphorylation of eEF2 at Thr^{56} in murine myotubes after HMB was added 2 h before PIF. Total eEF2 was used as a loading control. The densitometric analysis was the average of 3 separate Western blots and represents the ratio of phosphorylated to total forms of eEF2. Differences from control are shown as $P < 0.05$ (a), while differences from HMB alone are shown as $P < 0.01$ (e).

DISCUSSION

Previous studies (30) have suggested that PIF inhibits protein synthesis in muscle by reducing translational efficiency. Initiation of protein translation is controlled at the following two steps: binding of initiator methionyl-tRNA to the 40S ribosomal subunit and binding of mRNA to the 43S ribosomal subunit. PIF has been shown (10) to block the first step through an increase in phosphorylation of eIF2α, which competes with the guanine nucleotide exchange factor eIF2B for conversion of eIF2·GDP to eIF2·GTP, thus inhibiting translation initiation (29). The mechanism by which PIF produces this effect is through autophosphorylation of PKR at multiple sites, leading to activation (37). Activation of PKR by PIF not only reduces translational efficiency but also increases protein degradation (10). Although HMB alone had no effect on the basal level of phosphorylation of PKR and eIF2α, this study has shown it to block activation of PKR by PIF and the subsequent phosphorylation of eIF2α. This is the first study to show inhibition of both phosphorylation of PKR and eIF2α by an amino acid, although a decreased phosphorylation of eIF2α has been observed in leucine-starved myoblasts (36). These results would explain the ability of HMB to attenuate both the depression in protein synthesis in skeletal muscle and the increase in protein degradation, through the ubiquitin-proteasome pathway, in mice bearing the MAC16 tumor (33). The ability of HMB to attenuate phosphorylation of PKR and eIF2α in myotubes treated with PIF is at least partly responsible for its ability to

attenuate the depression in protein synthesis, since the eIF2α phosphatase inhibitor Salubrinal was able to reverse this effect. The mechanism by which HMB attenuates activation of PKR in the presence of PIF is not known but is likely to involve signaling pathways involved in the activation process, since when added alone it had no effect on phosphorylation of PKR. PKR is normally activated by viral double-strand RNA, but it can also be activated by polyanions and protein activators (25). The β-amyloid peptide of Alzheimer's disease also causes activation of PKR through calcium release from the endoplasmic reticulum and activation of caspase-8 and subsequently caspase-3 (35). Activation of caspase-3 has been shown to be an initial step in the loss of muscle proteins through the ubiquitin-proteasome pathway (9) and is known to be activated by PIF in murine myotubes, together with caspsases-8 and -9 (31).

HMB alone stimulated phosphorylation of mTOR and stimulated protein synthesis in myotubes in the absence of a catabolic signal (PIF). The stimulation of protein synthesis was also attenuated by rapamycin, confirming the importance of the mTOR pathway in this action. mTOR regulates phosphorylation of $p70^{S6k}$ and phosphorylation of 4E-BP1 through a common effector, which could be mTOR itself, or an mTOR-controlled downstream element, such as protein phosphatase (15). HMB alone caused increased phosphorylation of $p70^{S6k}$ and 4E-BP1. PIF alone also caused short-term (2 h) phosphorylation of both mTOR and $p70^{S6k}$, but in the presence of HMB phosphorylation of both mTOR and $p70^{S6k}$ continued throughout the course of measurement (6 h). This transient activation of the mTOR/$p70^{S6k}$ pathway by PIF may be necessary for increased synthesis of proteasome subunits and ubiquitin ligases required for protein degradation, since translation initiation will be attenuated by the increased phosphorylation of eIF2α. mTOR alone does not fully account for leucine stimulation of muscle protein synthesis, which may arise through direct activation of eIF4G. Leucine has been shown to stimulate phosphorylation of eIF4G, independent of mTOR, increasing the availability of eIF4G for eIF4E and increasing the formation of the eIF4E-eIF4G complex and protein synthesis (6).

It has previously been shown that multiple Ser/Thr residues in 4E-BP1 are phosphorylated in a hierarchical manner with phosphorylation of $Thr^{37/46}$ followed by Thr^{70}, and Ser^{65} is phosphorylated last (13). A combination of phosphorylation events is necessary to dissociate 4E-BP1 from eIF4E. In the current study, an antibody that recognizes phosphorylation at $Thr^{37/46}$ has been used, and this has shown an increase in the presence of HMB and HMB plus PIF, with a corresponding decrease in the amount of 4E-BP1 associated with eIF4E. In addition, there was an increase in both the phosphorylation of eIF4G and the amount of eIF4G associated with eIF4E, indicative of increased levels of the eIF4F complex. The interaction between eIF4E and eIF4G is regulated in part by the phosphorylation of eIF4G. Phosphorylation of eIF4G has been shown to be increased almost twofold by leucine in nonseptic but not in septic rats (19).

Clinical evaluation of HMB has used a combination with the amino acids L-arginine and L-glutamine (22). We have previously shown a significant stimulation of protein synthesis in the gastrocnemius muscle of weight-losing mice bearing the MAC16 tumor administered casein in combination with leucine, arginine, and methionine (32). In this study, both arginine and glutamine partially attenuated the depression of protein synthesis and RNA content of myotubes in the presence of PIF but did not significantly enhance the effect of HMB.

Another target of mTOR is the elongation factor eEF2 (27). However, the phosphorylation status of $p70^{S6k}$ and eEF2 is regulated in opposing directions by mTOR signaling. Phosphorylation of eEF2 inhibits its activity by preventing its interaction with the ribosome. Insulin induces dephosphorylation of eEF2 through a decrease in activity of eEF2 kinase, and this is blocked by rapamycin (40). eEF2 kinase is phosphorylated and inactivated by $p70^{S6k}$, which is activated by insulin through mTOR. Amino acids have also been shown to cause inhibition of eEF2 kinase, resulting in an increase in the proportion of eEF2 in the active, dephosphorylated form (18). Leucine starvation has been shown to increase phosphorylation of eEF2 at Thr^{56} (36), and the current study shows that HMB also decreases it. PIF elicited a small increase in the phosphorylation of eEF2 that did not reach significance, but this was not attenuated by HMB.

Thus HMB attenuates the inhibition of protein synthesis in muscle by multiple mechanisms, including downregulation of eIF2a phosphorylation through an effect on PKR and upregulation of the mTOR/$p70^{S6k}$ pathway, resulting in increased phosphorylation of 4E-BP1 and an increase in the active eIF4G·eIF4E complex. Many of these features are also shared with leucine. However, HMB is more potent than leucine in attenuating the development of cachexia (32, 34) and is better tolerated by oral administration.

ACKNOWLEDGMENTS

We thank M. Wynter and W. Fleary for performing the animal experiment.

GRANTS

This work was supported by Ross Nutrition/Abbott Laboratories.

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