

# Mechanism of the Attenuation of Proteolysis-Inducing Factor Stimulated Protein Degradation in Muscle by $\beta$ -Hydroxy- $\beta$ -Methylbutyrate

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## ABSTRACT

The leucine metabolite  $\beta$ -hydroxy- $\beta$ -methylbutyrate (HMB) prevents muscle protein degradation in cancer-induced weight loss through attenuation of the ubiquitin-proteasome proteolytic pathway. To investigate the mechanism of this effect, the action of HMB on protein breakdown and intracellular signaling leading to increased proteasome expression by the tumor factor proteolysis-inducing factor (PIF) has been studied *in vitro* using murine myotubes as a surrogate model of skeletal muscle. A comparison has been made of the effects of HMB and those of eicosapentaenoic acid (EPA), a known inhibitor of PIF signaling. At a concentration of 50  $\mu\text{mol/L}$ , EPA and HMB completely attenuated PIF-induced protein degradation and induction of the ubiquitin-proteasome proteolytic pathway, as determined by the “chymotrypsin-like” enzyme activity, as well as protein expression of 20S proteasome  $\alpha$ - and  $\beta$ -subunits and subunit p42 of the 19S regulator. The primary event in PIF-induced protein degradation is thought to be release of arachidonic acid from membrane phospholipids, and this process was attenuated by EPA, but not HMB, suggesting that HMB might act at another step in the PIF signaling pathway. EPA and HMB at a concentration of 50  $\mu\text{mol/L}$  attenuated PIF-induced activation of protein kinase C and the subsequent degradation of inhibitor  $\kappa\text{B}\alpha$  and nuclear accumulation of nuclear factor  $\kappa\text{B}$ . EPA and HMB also attenuated phosphorylation of p42/44 mitogen-activated protein kinase by PIF, thought to be important in PIF-induced proteasome expression. These results suggest that HMB attenuates PIF-induced activation and increased gene expression of the ubiquitin-proteasome proteolytic pathway, reducing protein degradation.

## INTRODUCTION

Loss of myofibrillar proteins from skeletal muscle has a negative effect on the quality and quantity of life of the cancer patient. Decreased protein synthesis and increased protein catabolism contribute to muscle atrophy (1), but anabolic stimuli alone cannot increase muscle mass and require concomitant attenuation of the increased protein degradation (2). The major system for protein degradation in cancer-induced weight loss is thought to be the ubiquitin-proteasome pathway (3).

In a previous report,<sup>1</sup> we have shown protein degradation in skeletal muscle of mice bearing the MAC16 cachexia-inducing tumor to be attenuated by  $\beta$ -hydroxy- $\beta$ -methylbutyrate (HMB) by inhibition of the increased expression of the ubiquitin-proteasome pathway. Although some cytokines, such as tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), have been shown to induce expression of the ubiquitin-proteasome pathway in skeletal muscle (4), there is no evidence for cytokine involvement in the MAC16 cachexia model (5). Instead, a tumor product, proteolysis-inducing factor (PIF), appears to be responsible for the loss of muscle mass (6), also through induction of key regulatory elements for proteasome proteolysis (7). PIF expression

has been found in carcinomas of the prostate (8), colon, lung, esophagus, liver, and pancreas when weight loss is apparent (9) but not in normal prostate tissue or stromal cells. It previously has been shown that the action of PIF is attenuated by the polyunsaturated fatty acid eicosapentaenoic acid (EPA; ref. 10), which suppresses protein degradation in skeletal muscle through inhibition of the increased expression of the ubiquitin-proteasome proteolytic pathway (11). The action of EPA is manifested by interference with the PIF signaling pathway in muscle cells, leading to increased proteasome expression.

The full details of this pathway still have to be elucidated, but the first step involves the release of arachidonic acid from membrane phospholipids and the rapid metabolism to a range of eicosanoids, of which 15-hydroxyeicosatetraenoic acid (15-HETE) alone is capable of inducing protein degradation (10), by increasing proteasome expression (12). EPA attenuates the release of arachidonic acid and its metabolism to 15-HETE (10). Induction of proteasome expression by PIF (13) and 15-HETE (12) also was associated with an increased nuclear accumulation of the transcription factor nuclear factor  $\kappa\text{B}$  (NF $\kappa\text{B}$ ) and transitory depletion of inhibitor  $\kappa\text{B}\alpha$  (I $\kappa\text{B}\alpha$ ) from the cytosol, a process also attenuated by EPA. This process requires protein kinase C (PKC), which probably is involved in the phosphorylation and degradation of I $\kappa\text{B}\alpha$ , necessary for the release of NF $\kappa\text{B}$  from its inactive cytosolic complex (14).

This study examines the effect of HMB on PIF-induced protein degradation and signaling pathways in murine myotubes, in comparison with EPA, to determine the mechanism of the attenuation of the increased expression of the ubiquitin-proteasome proteolytic pathway observed in a murine model of cancer-induced weight loss.

## MATERIALS AND METHODS

**Materials.** L-[2,6-<sup>3</sup>H]Phenylalanine (specific activity, 2.00 TBq/mmol) was purchased from Amersham Biosciences (Piscataway, NJ), and [5,6,8,9,11,12,14,15-<sup>3</sup>H]arachidonic acid (specific activity, 3.7 TBq/mmol) was purchased from Tocris Cookson (Avonmouth, United Kingdom). EPA (99%) as free acid was purchased from Sigma-Aldridge (St. Louis, MO). EPA was used at a concentration of 50  $\mu\text{mol/L}$ , which previously has been shown to be effective in attenuating the action of PIF (10, 12, 13). HMB (as the calcium salt) was obtained from Organic Technologies Inc. (Coshocton, OH). Mouse monoclonal antibodies to 20S proteasome subunits  $\alpha$ 1, 2, 3, 5, 6, and 7 (clone MCP231), 20S proteasome subunit  $\beta$ 3 (HC10), and 19S regulator ATPase subunit Rpt 4 (S106, p42; clone p42-23) were purchased from BIOMOL International (Plymouth Meeting, PA). Rabbit polyclonal antisera to murine I $\kappa\text{B}\alpha$ , phospholipase A<sub>2</sub> (PLA<sub>2</sub>), type V1, and PKC $\alpha$  were from Calbiochem (La Jolla, CA). Mitogen-activated protein kinase (MAPK) and the phosphorylated (active) forms were detected with anti-extracellular signal-regulated kinase (ERK) 1 and 2 [pTpY<sup>185/187</sup>] nonphospho-specific and phospho-specific rabbit polyclonal antisera (Biosource International, Camarillo, CA). Rabbit polyclonal antisera to mouse actin were from Sigma-Aldridge. Peroxidase-conjugated goat antirabbit and rabbit antimouse secondary antibodies were from Dako (Carpinteria, CA). Hybond nitrocellulose membranes and enhanced chemiluminescence were from Amersham Biosciences. Electrophoretic mobility shift (EMSA) gel shift assay kits were from Panomics (Redwood City, CA). Fetal calf serum, horse serum (HS), and Dulbecco's modified Eagle's medium (DMEM) were purchased from Life Technologies, Inc. (Rockville, MD).

**Cell Culture.** C<sub>2</sub>C<sub>12</sub> myotubes were routinely passaged in DMEM supplemented with 10% fetal calf serum, glutamine, and 1% penicillin-streptomycin

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under an atmosphere of 10% CO<sub>2</sub> in air at 37°C. Myotubes were formed by allowing confluent cultures to differentiate in DMEM containing 2% HS, with medium changes every 2 days.

**Purification of Proteolysis-Inducing Factor.** PIF was purified from solid MAC16 tumors (15) excised from mice with a weight loss of 20 to 25%. Tumors were homogenized in 10 mmol/L Tris-HCl (pH 8.0) containing 0.5 mmol/L phenylmethylsulfonyl fluoride, 0.5 mmol/L EGTA, and 1 mmol/L dithiothreitol at a concentration of 5 mL/g tumor. Solid ammonium sulfate was added to 40% w/v, and the supernatant, after removal of the ammonium sulfate, was subjected to affinity chromatography using anti-PIF monoclonal antibody coupled to a solid matrix as described previously (16). The immunogenic fractions were concentrated and used for further studies.

**Measurement of Total Protein Degradation.** Myotubes in six-well multidishes were labeled with L-[2,6-<sup>3</sup>H]phenylalanine (0.67 mCi/mmol) for 24 hours in 2 mL DMEM containing 2% HS. They then were washed three times in PBS, followed by a 2-hour incubation at 37°C in DMEM without phenol red until no more radioactivity appeared in the supernatant. Cells then were preincubated for 2 hours with and without EPA or HMB and then for 24 hours with PIF in fresh DMEM without phenol red to prevent quenching of counts and in the presence of 2 mmol/L cold phenylalanine to prevent reincorporation of radioactivity. The amount of radioactivity released into the medium was expressed as a percentage of control cultures not exposed to PIF.

**Measurement of Arachidonate Release.** Myotubes in six-well multidishes containing 2 mL DMEM with 2% HS were labeled for 24 hours with 10 μmol/L arachidonic acid (containing 1 μCi [<sup>3</sup>H]arachidonate/mL; ref. 10). Cells then were washed extensively with PBS to remove traces of unincorporated [<sup>3</sup>H]arachidonate, and either EPA or HMB was added 2 hours before PIF. After an additional 24 hours, 1 mL of medium was removed to determine the radioactivity released.

**Measurement of Proteasome Activity.** The functional activity of the β-subunits of the proteasome was determined as the “chymotrypsin-like” enzyme activity determined fluorimetrically according to the method of Orino *et al.* (17). Myotubes were exposed to PIF for 24 hours with or without EPA or HMB added 2 hours before PIF, and enzyme activity was determined in a supernatant fraction (13) by the release of aminomethyl coumarin (AMC) from succinyl-LLVY-AMC (0.1 mmol/L) in the presence or absence of the specific proteasome inhibitor lactacystin (10 μmol/L; ref. 18). Only lactacystin-suppressible activity was considered to be proteasome specific. Activity was adjusted for the protein concentration of the sample determined using the Bradford assay (Sigma Chemical Co., St. Louis, MO) using bovine serum albumin as standard.

**Western Blot Analysis.** Cytosolic protein (2 to 5 μg) obtained for the aforementioned assay was resolved on 10% SDS-PAGE and transferred to 0.45 μm nitrocellulose membrane, which had been blocked with 5% Marvel in PBS, at 4°C overnight. The primary antibodies were used at a dilution of 1:100 (antiactin), 1:500 (anti-ERK1/2, PKCα, and PLA<sub>2</sub>), 1:1000 (anti-20S proteasome β-subunit and IκBα), 1:1500 (anti-20S proteasome α-subunit), or 1:5000 (anti-p42), whereas the secondary antibodies were used at a dilution of 1:2000. Incubation was carried out for 2 hours at room temperature, and development was by enhanced chemiluminescence. Loading was quantitated by actin concentration.

**Electrophoretic Mobility Shift.** DNA binding proteins were extracted from myotubes by the method of Andrews and Faller (19), which uses hypotonic lysis followed by high salt extraction of nuclei. The EMSA binding assay was carried out according to the manufacturer's instructions.

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

## RESULTS

Because protein degradation and activation of the ubiquitin-proteasome proteolytic pathway in mice bearing the MAC16 tumor are thought to be mediated by PIF (6), mechanistic studies on the effect of HMB on protein degradation were carried out in murine myotubes treated with PIF. PIF induced total protein breakdown with a typical bell-shaped dose-response curve as reported previously (10) with a maximal effect at 4 nmol/L (Fig. 1A). The effect of HMB has been compared with EPA, and the data in Fig. 1A show that at a concentration of 50 μmol/L both were equally effective in attenuating PIF-induced protein degradation. There also was some attenuation at

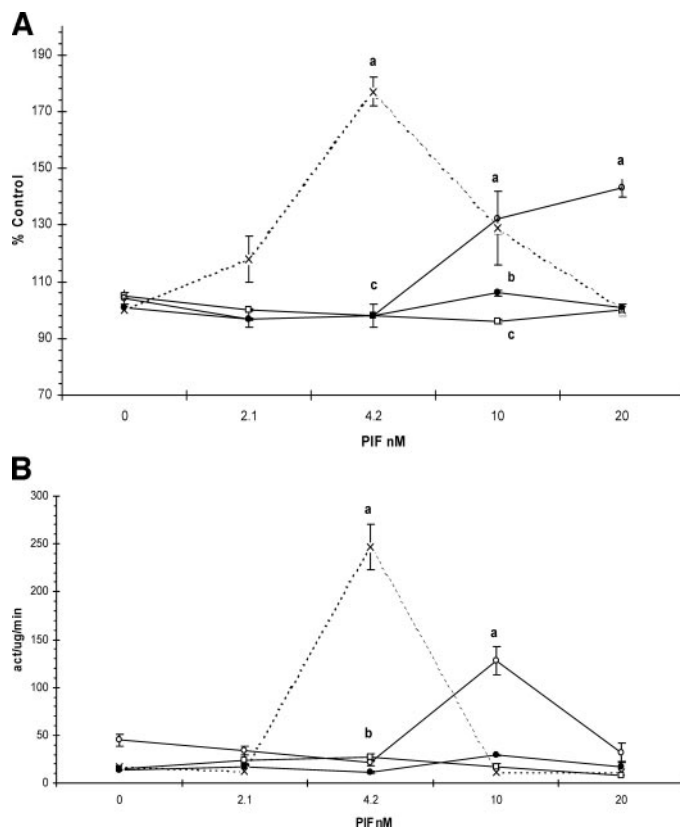


Fig. 1. A, the effect of PIF on total protein degradation in C<sub>2</sub>C<sub>12</sub> myotubes in the absence (X) or presence of either 50 μmol/L EPA (□) or 25 μmol/L (○) or 50 μmol/L (●) HMB. Measurements were made 24 hours after the addition of PIF and are shown as mean ± SE, where *n* = 9. Differences from control in the absence of PIF are indicated as *a* (*P* < 0.005), whereas differences from PIF alone in the presence of EPA or HMB are shown as *b* (*P* < 0.01) and *c* (*P* < 0.005). B, chymotryptic activity of soluble extracts of murine myotubes treated with PIF in the absence or presence of EPA (50 μmol/L) or HMB (25 or 50 μmol/L). The symbols are the same as in A. The results are shown as mean ± SE, where *n* = 9. Differences from control are shown as *a* (*P* < 0.001), whereas differences in the presence of EPA or HMB are shown as *b* (*P* < 0.001).

25 μmol/L HMB at low, but not at high, concentrations of PIF, which appeared to potentiate the effect of PIF alone.

To determine whether HMB attenuated protein degradation induced by PIF through the ubiquitin-proteasome proteolytic pathway, the expression and functional activity of this pathway were assessed. PIF induced an increase in “chymotrypsin-like” enzyme activity, the predominant proteolytic activity of the β-subunits of the proteasome, which was maximal at 4.2 nmol/L (Fig. 1B), as was protein degradation (Fig. 1A). The effect of PIF was completely attenuated by 50 μmol/L EPA and 25 and 50 μmol/L HMB, although 25 μmol/L HMB produced an increase in “chymotrypsin-like” enzyme activity at 10 nmol/L PIF as observed with protein degradation (Fig. 1A). A similar effect was observed on expression of proteasome 20S α-subunits, β-subunits, and p42, an ATPase subunit of the 19S regulator that promotes ATP-dependent association of the 20S proteasome with the 19S regulator to form the 26S proteasome (ref. 20; Fig. 2). In all of the cases, expression was increased by PIF at 4.2 and 10 nmol/L, and this was attenuated by EPA and HMB at 50 μmol/L but not at 25 μmol/L. These results confirm that HMB attenuates protein degradation through an effect on PIF induction of the ubiquitin-proteasome pathway.

To determine the mechanism by which HMB inhibits PIF-induced proteasome expression, a comparison has been made with EPA on key signaling pathways leading to an increased gene expression. As observed previously (10), the PIF-induced release of arachidonic acid (AA) was attenuated by EPA (Fig. 3), but HMB at 50 μmol/L had no

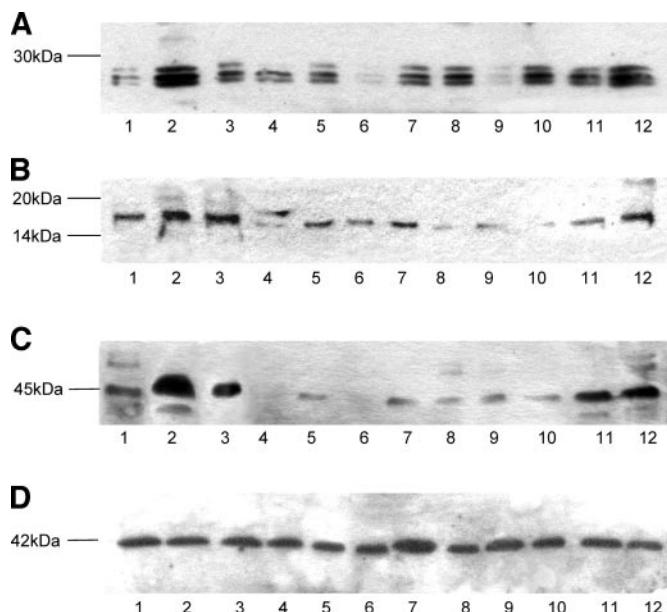


Fig. 2. The effect of EPA and HMB on PIF induction of 20S proteasome  $\alpha$ -subunit (A),  $\beta$ -subunit (B), and p42 (C). The actin loading control is shown in D. Western blot analyses of soluble extracts of  $C_2C_{12}$  myotubes 24 hours after treatment with PIF alone (Lanes 1 through 3) or with PIF in the presence of 50  $\mu\text{mol/L}$  EPA (Lanes 4 through 6), 50  $\mu\text{mol/L}$  HMB (Lanes 7 through 9), or 25  $\mu\text{mol/L}$  HMB (Lanes 10 through 12) at a concentration of PIF of 4.2 nmol/L (Lanes 2, 5, 8, and 11) or 10 nmol/L (Lanes 3, 6, 9, and 12). Control cultures received PBS (Lane 1), 50  $\mu\text{mol/L}$  EPA (Lane 4), 50  $\mu\text{mol/L}$  HMB (Lane 7), or 25  $\mu\text{mol/L}$  HMB (Lane 10). The blots shown are representative of three separate experiments.

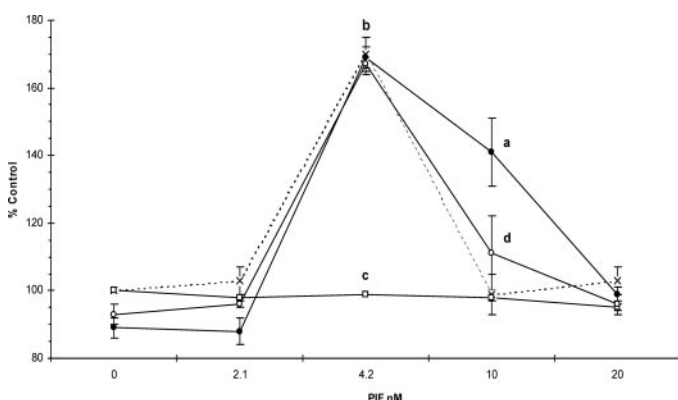


Fig. 3. Effect of PIF on release of [ $^3\text{H}$ ]arachidonate from  $C_2C_{12}$  myotubes without (X) or with coincubation with 50  $\mu\text{mol/L}$  EPA (■), 50  $\mu\text{mol/L}$  HMB (□), or 25  $\mu\text{mol/L}$  HMB (●). Results are shown as mean  $\pm$  SE, where  $n = 9$ . Differences from control are shown as a ( $P < 0.01$ ) and b ( $P < 0.001$ ), whereas differences in the presence of EPA are shown as c ( $P < 0.001$ ) and from 25  $\mu\text{mol/L}$  HMB as d ( $P < 0.05$ ).

effect on this process, suggesting that it acts farther down the pathway. At 25  $\mu\text{mol/L}$ , HMB caused a significant increase in release of AA when combined with 10 nmol/L PIF, suggesting a mechanism for the increased protein degradation (Fig. 1A) and chymotrypsin-like enzyme activity (Fig. 1B) and proteasome expression (Fig. 2) at this concentration. PIF caused an up-regulation of calcium-independent cytosolic  $\text{PLA}_2$  at 4.2 and 10 nmol/L (Fig. 4), corresponding with the maximal effect on AA release (Fig. 3). This effect was attenuated by EPA, but not HMB, at both concentrations. This suggests that HMB attenuates PIF-induced proteasome expression at a step in the intracellular signaling pathway after  $\text{PLA}_2$ . Experiments using mutants of PKC and inhibitors of this enzyme show that this forms a central mediator of intracellular signaling by PIF (14). PIF stimulates translocation of  $\text{PKC}\alpha$  from the cytoplasm to the plasma membrane (Fig. 5), resulting in activation, with a maximum effect at 4.2 nmol/L PIF,

as with protein degradation (Fig. 1). This process was effectively attenuated by EPA and HMB at 50  $\mu\text{mol/L}$ , whereas HMB was less effective at 25  $\mu\text{mol/L}$  (Fig. 5). This suggests that HMB attenuates PIF-induced protein degradation through inhibition of PKC.

Activation of PKC has been shown to activate ERK and subsequently MAPK pathways (21) and that this is involved in PIF-induced proteasome expression (22). The effect of EPA and HMB on this process is shown in Fig. 6. There was no effect on total ERK1/2 (Fig. 6A), whereas PIF induced an increased phosphorylation of p42/44, which was maximal at 4.2 nmol/L, and this effect was completely attenuated by EPA and HMB at 50  $\mu\text{mol/L}$  but not by HMB at 25  $\mu\text{mol/L}$  (Fig. 6B). The ability of HMB to attenuate ERK1/2 phosphorylation suggests that this step is after the activation of PKC and also may be important in inhibition of PIF-induced proteasome expression by HMB.

As discussed previously, PIF induces degradation of  $\text{I}\kappa\text{B}\alpha$  and stimulates nuclear accumulation of  $\text{NF}\kappa\text{B}$ , and this process has been

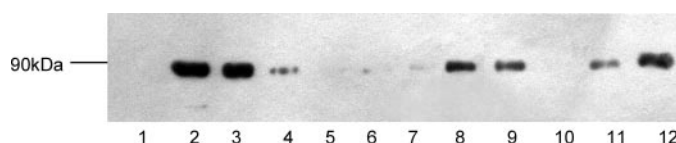


Fig. 4. Effect of PIF on the expression of  $\text{PLA}_2$  in the absence (Lanes 1 through 3) or in the presence of 50  $\mu\text{mol/L}$  EPA (Lanes 4 through 6), 50  $\mu\text{mol/L}$  HMB (Lanes 7 through 9), or 25  $\mu\text{mol/L}$  HMB (Lanes 10 through 12) at 4.2 nmol/L PIF (Lanes 2, 5, 8, and 11) or 10 nmol/L PIF (Lanes 3, 6, 9, and 12). Control cultures received PBS (Lane 1), 50  $\mu\text{mol/L}$  EPA (Lane 4), 50  $\mu\text{mol/L}$  HMB (Lane 7), or 25  $\mu\text{mol/L}$  HMB (Lane 10). The blot shown is representative of three separate experiments.

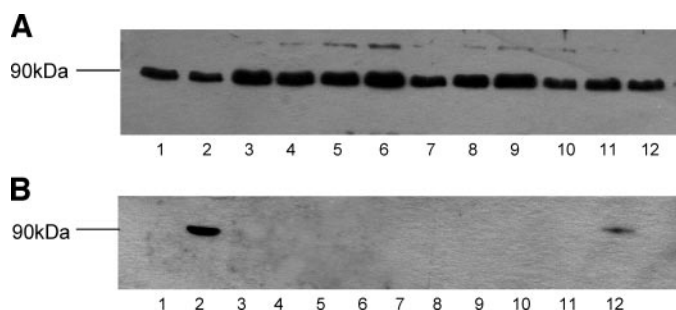


Fig. 5. Western blot analysis of the effect of PIF on cytoplasmic (A) and membrane-bound (B)  $\text{PKC}\alpha$  in murine myotubes. Cells were treated with PIF alone (Lanes 1 through 3) or with PIF in the presence of 50  $\mu\text{mol/L}$  EPA (Lanes 4 through 6), 50  $\mu\text{mol/L}$  HMB (Lanes 7 through 9), or 25  $\mu\text{mol/L}$  HMB (Lanes 10 through 12) at 4.2 nmol/L (Lanes 2, 5, 8, and 11) or 10 nmol/L PIF (Lanes 3, 6, 9, and 12). Control cells received PBS (Lane 1), 50  $\mu\text{mol/L}$  EPA (Lane 4), 50  $\mu\text{mol/L}$  HMB (Lane 7), or 25  $\mu\text{mol/L}$  HMB (Lane 10). The blots shown are representative of three separate experiments.

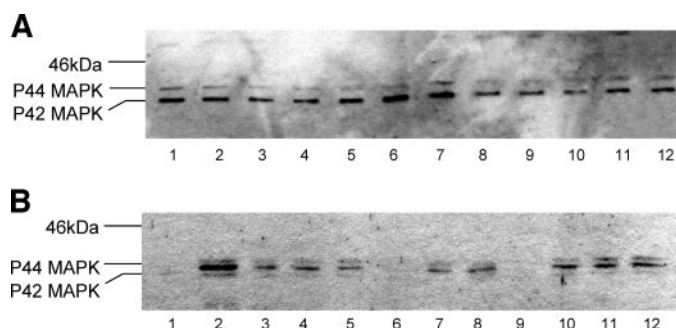


Fig. 6. Western blot analysis of total ERK1/2 (p44 and p42; A) and active (phosphorylated) ERK1/2 (B) in soluble extracts of murine myotubes treated with PIF alone (Lanes 1 through 3) or with PIF in the presence of 50  $\mu\text{mol/L}$  EPA (Lanes 4 through 6), 50  $\mu\text{mol/L}$  HMB (Lanes 7 through 9), or 25  $\mu\text{mol/L}$  HMB (Lanes 10 through 12) at a PIF concentration of 4.2 nmol/L (Lanes 2, 5, 8, and 11) or 10 nmol/L (Lanes 3, 6, 9, and 12). Control cells received PBS (Lane 1), 50  $\mu\text{mol/L}$  EPA (Lane 4), 50  $\mu\text{mol/L}$  HMB (Lane 7), or 25  $\mu\text{mol/L}$  HMB (Lane 10). The blots shown are representative of three separate experiments.



shown to be attenuated by 50  $\mu\text{mol/L}$  EPA (13). The results in Fig. 7A show HMB at 50  $\mu\text{mol/L}$  to effectively attenuate I $\kappa$ B $\alpha$  degradation in the presence of PIF in murine myotubes and to prevent nuclear accumulation of NF $\kappa$ B (Fig. 7D). Only partial inhibition of binding of NF $\kappa$ B to DNA was observed when HMB was used at a concentration of 25  $\mu\text{mol/L}$  (Fig. 7C). These results suggest that the overall effect of HMB is similar to that of EPA in preventing movement of NF $\kappa$ B into the nucleus with concomitant activation of gene expression.

## DISCUSSION

HMB is a metabolite of leucine, which is thought to be responsible for inhibiting muscle proteolysis and modulating protein turnover *in vitro* and *in vivo* (23). Oral supplementation with the combination of HMB, arginine, and glutamine increased muscle mass and body weight in patients with cancer (24) and AIDS-associated wasting (25). It was thought that arginine and glutamine supplementation enhanced net protein synthesis, whereas supplementation with HMB minimized protein breakdown, although this has not been proven, and the mechanism by which HMB produced the effect is not known. This study has examined the effect of HMB on PIF-induced protein degradation in murine myotubes. PIF-induced protein degradation previously has been shown to

result from an increased activity and expression of the regulatory components of the ubiquitin-proteasome proteolytic pathway (7, 26), and HMB has been shown to effectively attenuate protein degradation through an effect on signaling pathways leading to up-regulation of proteasome expression. This mechanism may be applicable to other forms of protein degradation because there is evidence to suggest a commonality in signaling pathways for all of the types of muscle atrophy (27).

In many respects the effect of HMB is similar to that of EPA, which also attenuates the increased proteasome proteolysis in cancer-induced weight loss (11). Both are equipotent, and both inhibit PIF-induced signaling pathways. PIF previously has been reported to induce activation of PLA<sub>2</sub> (22), with release of arachidonic acid from membrane phospholipids, as a primary event in the signaling cascade. EPA has been shown to block the release of AA and the conversion to 15-HETE (10), whereas HMB has no effect on this process. Low concentrations (25  $\mu\text{mol/L}$ ) of HMB combined with higher concentrations of PIF (10 nmol/L) appear to increase release of AA, which may account for the stimulation of protein breakdown and expression of the ubiquitin-proteasome pathway at these concentrations. PIF induced protein degradation with a parabolic dose-response curve as reported previously (10, 12, 13). The shape of the

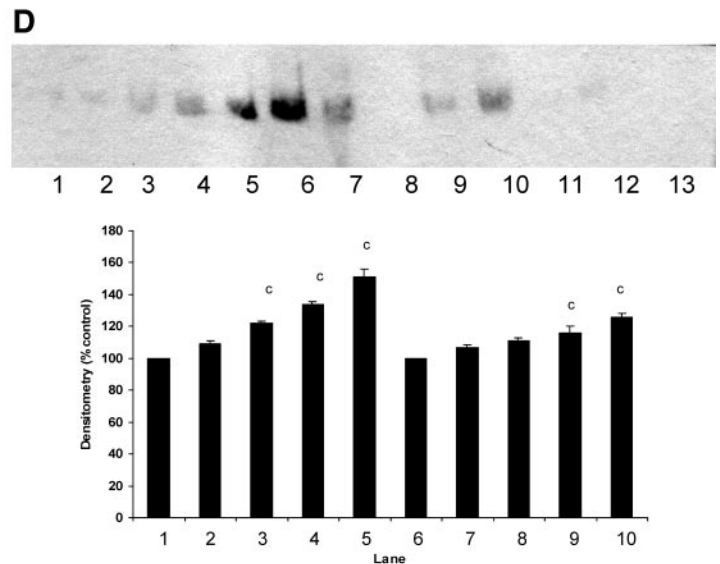
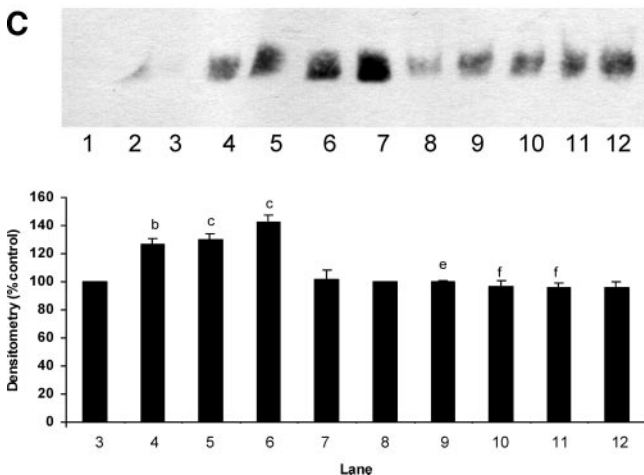
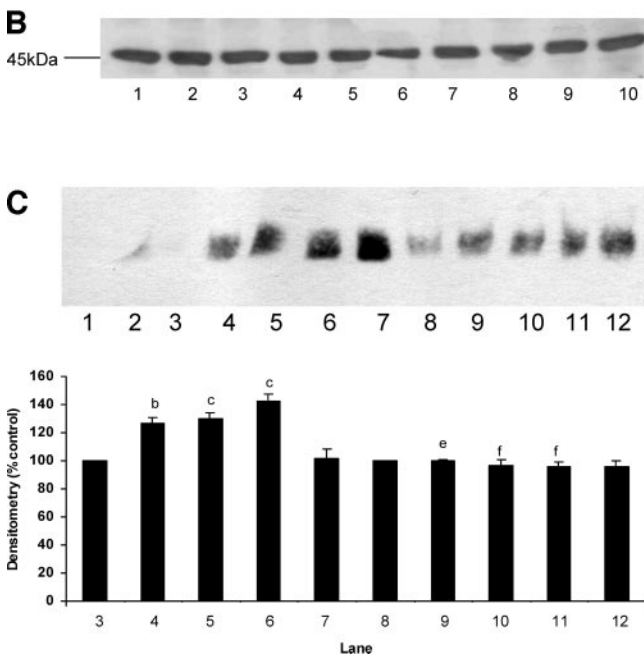
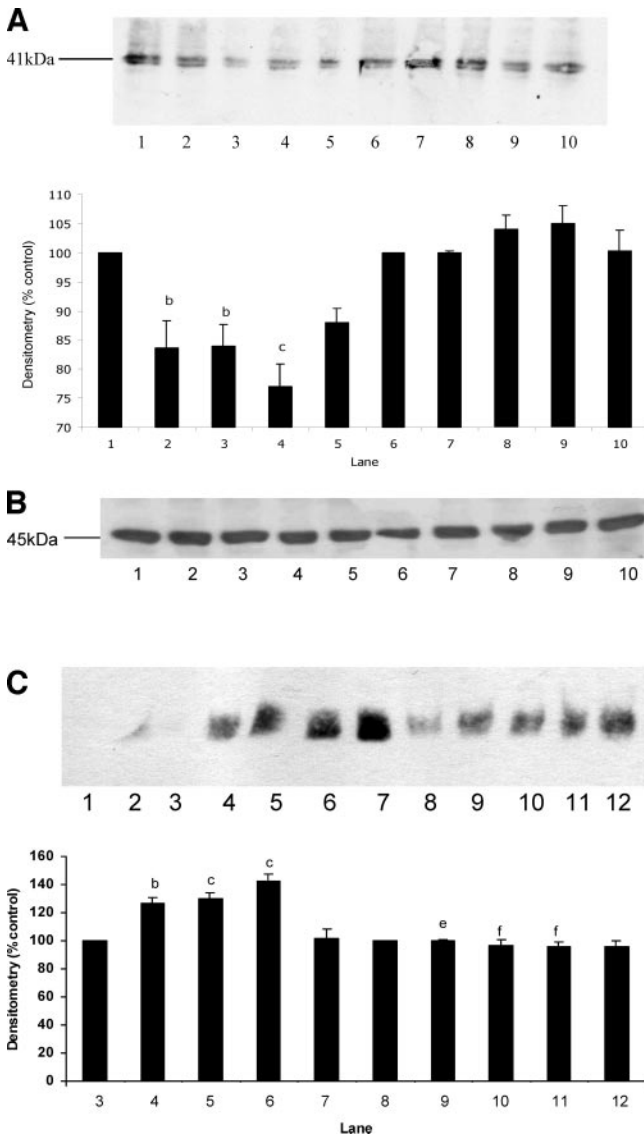


Fig. 7. Effect of exposure of C<sub>2</sub>C<sub>12</sub> myotubes for 30 minutes on cytosolic levels of I $\kappa$ B $\alpha$  (A), determined by Western blot analysis, and activation of NF $\kappa$ B binding to DNA, as determined by EMSA (C and D). B, the actin loading control for the blot shown in A. For A and B, myotubes were treated with PIF alone (Lanes 1 through 5) or with PIF in the presence of 50  $\mu\text{mol/L}$  HMB (Lanes 6 through 10) at a concentration of 0 (Lanes 1 and 6), 2.1 (Lanes 2 and 7), 4.2 (Lanes 3 and 8), 10.5 (Lanes 4 and 9), or 16.8 nmol/L PIF (Lanes 5 and 10). The densitometric analysis is an average of three replicate blots. Differences from 0 nmol/L PIF are shown as b ( $P < 0.01$ ) and c ( $P < 0.001$ ). In C and D, myotubes were treated with 0 (Lanes 3 and 8), 2.1 (Lanes 4 and 9), 4.2 (Lanes 5 and 10), 10.5 (Lanes 6 and 11), and 16.8 nmol/L PIF (Lanes 7 and 12) in the absence (Lanes 3 to 7) or presence (Lanes 8 to 12) of 25  $\mu\text{mol/L}$  HMB (C) or 50  $\mu\text{mol/L}$  HMB (D). Lane 1 contains a 100-fold excess of unlabeled NF $\kappa$ B probe, whereas Lane 2 is a positive control for NF $\kappa$ B (supplied by the manufacturer of the kit).

dose-response curve is thought to result from the requirement of PKC in the signaling pathway (14). Overstimulation of PKC results in down-regulation of activity. The first step at which HMB inhibits PIF signaling appears to be through PKC. EPA also appears to block PKC, but this may be an indirect effect because AA and lipoxigenase metabolites have been shown to activate PKC (28). PIF also has been shown to activate phospholipase C (PLC), and this step also is involved in the increase in proteasome expression (22). Activation of phospholipase C would result in the generation of diacylglycerol, which also could induce translocation of PKC from the cytosol to the plasma membrane, resulting in the complete activation of the kinase. PKC appears to be important in the induction of proteasome expression by PIF because it was blocked by inhibitors of PKC and in myotubes transfected with a dominant-negative PKC $\alpha$ , which showed no activation of PKC $\alpha$  (14). Thus, the ability of HMB to block activation of PKC by PIF would explain the ability to inhibit proteasome expression and the subsequent increase in protein degradation. The mechanism of the inhibitory effect of HMB is not known; it may be direct or may be caused by inhibition of phospholipase C.

Activation of PKC may induce phosphorylation and activation of the I $\kappa$ B $\alpha$  kinase complex (29) responsible for the phosphorylation of I $\kappa$ B $\alpha$  at serine-32 and -36, leading to ubiquitination and subsequent degradation by the proteasome system. Degradation of I $\kappa$ B $\alpha$  releases NF $\kappa$ B, which then is able to enter the nucleus and stimulate gene expression by binding to its target elements. Induction of proteasome expression by PIF (13) and 15-HETE (12) appears to require NF $\kappa$ B because the NF $\kappa$ B inhibitor peptide SN50 also attenuated the increase in proteasome expression. Myotubes transfected with mutants at the serine phosphorylation sites of I $\kappa$ B, which are required for degradation, also were resistant to PIF-induced protein degradation and proteasome expression.<sup>2</sup> If HMB attenuates PIF-induced activation of PKC, then downstream signaling pathways, namely, degradation of I $\kappa$ B $\alpha$  and nuclear accumulation of NF $\kappa$ B, also would be inhibited, as was observed, thus explaining the ability of HMB to attenuate the PIF-induced increase in the ubiquitin-proteasome pathway.

The ERK/MAPK pathway also was shown to be important in PIF-induced proteasome expression (22). Thus, PIF induces phosphorylation of ERK1 and ERK2 at the same concentrations as those inducing proteasome expression, whereas a selective inhibitor of MAP/ERK kinase attenuated the PIF-induced activation of ERK1 and ERK2 and the induction of proteasome expression. The ERK/MAPK pathway is an intracellular transduction system mainly involved in the cellular response to growth factors and is activated through tyrosine kinase receptors, acting through small G proteins, such as Ras (30). It previously has been shown (20) that two tyrosine kinase inhibitors, genistein and tryptostin A23, attenuated the PIF-induced increase in proteasome activity, suggesting the involvement of tyrosine kinase in this process. The PKC pathway is linked to the MAPK pathway through multiple steps. Thus, stimulation of G-protein receptors can indirectly activate MAPK through the PKC pathway (31). It has been proposed that PKC $\alpha$  phosphorylates and activates Raf-1 kinase, a substrate not only for the  $\alpha$  isoforms but also for the other PKC isozymes,  $\beta$  and  $\gamma$  (32), and this in turn leads to activation of MAPK through the kinase cascade (33). The MAPK pathway may provide an alternative mechanism for proteasome expression in addition to NF $\kappa$ B.

Thus, HMB appears to be an effective agent for the management of muscle wasting in cancer-induced weight loss. HMB appears to exert its effect by attenuation of PIF-induced protein degradation mediated through the ubiquitin-proteasome pathway by inhibition of PKC, with resultant stabilization of the cytoplasmic I $\kappa$ B/NF $\kappa$ B complex.

## REFERENCES

- Baracos VE. Management of muscle wasting in cancer-associated cachexia. *Cancer* 2001;92:1669–77.
- Barber MD, Ross JA, Voss AC, Tisdale MJ, Fearon KCH. The effect of an oral nutritional supplement enriched with fish oil on weight-loss in patients with pancreatic cancer. *Br J Cancer* 1999;81:80–6.
- Bossola M, Muscaritoli M, Costelli P, et al. Increased muscle proteasome activity correlates with disease severity in gastric cancer patients. *Ann Surgery* 2003;237:384–9.
- Llovera M, Garcia-Martinez C, Agell N, Lopez-Soriano FJ, Argiles JM. TNF can directly induce the expression of ubiquitin-dependent proteolytic system in rat soleus muscles. *Biochem Biophys Res Commun* 1997;230:238–41.
- Mulligan HD, Mahony SM, Ross JA, Tisdale MJ. Weight loss in a murine cachexia model is not associated with the cytokines tumour necrosis factor- $\alpha$  or interleukin-6. *Cancer Lett* 1992;65:239–43.
- Lorite MJ, Thompson MG, Drake JL, Carling G, Tisdale MJ. Mechanism of muscle protein degradation induced by a cancer cachectic factor. *Br J Cancer* 1998;78:850–6.
- Lorite MJ, Smith HJ, Arnold JA, Morris A, Thompson MG, Tisdale MJ. Activation of ATP-ubiquitin-dependent proteolysis in skeletal muscle in vivo and murine myoblasts in vitro by a proteolysis-inducing factor (PIF). *Br J Cancer* 2001;85:297–302.
- Wang Z, Corey E, Hass GM, et al. Expression of the human cachexia-associated protein (HCAP) in prostate cancer and in a prostate cancer animal model of cachexia. *Int J Cancer* 2003;105:123–9.
- Cabal-Manzano R, Bhargava P, Torres-Duarte A, Marshall J, Bhargava P, Wainer IW. Proteolysis-inducing factor is expressed in tumours of patients with gastrointestinal cancers and correlates with weight loss. *Br J Cancer* 2001;84:1599–601.
- Smith HJ, Lorite MJ, Tisdale MJ. Effect of a cancer cachectic factor on protein synthesis/degradation in murine C<sub>2</sub>C<sub>12</sub> myoblasts: modulation by eicosapentaenoic acid. *Cancer Res* 1999;59:5507–13.
- Whitehouse AS, Smith HJ, Drake JL, Tisdale MJ. Mechanism of attenuation of skeletal muscle protein catabolism in cancer cachexia by eicosapentaenoic acid. *Cancer Res* 2001;61:3604–9.
- Whitehouse AS, Khal J, Tisdale MJ. Induction of protein catabolism in myotubes by 15(S)-hydroxyeicosatetraenoic acid through increased expression of the ubiquitin-proteasome pathway. *Br J Cancer* 2003;89:737–45.
- Whitehouse AS, Tisdale MJ. Increased expression of the ubiquitin-proteasome pathway in murine myotubes by proteolysis-inducing factor (PIF) with activation of the transcription factor NF- $\kappa$ B. *Br J Cancer* 2003;89:1116–22.
- Smith HJ, Wyke SM, Tisdale MJ. Role of protein kinase C and NF- $\kappa$ B in proteolysis-inducing factor-induced proteasome expression in C<sub>2</sub>C<sub>12</sub> myotubes. *Br J Cancer* 2004;90:1850–7.
- Todorov P, Cariuk P, McDevitt T, Coles B, Fearon KCH, Tisdale M. Characterization of a cancer cachectic factor. *Nature* 1996;379:739–42.
- Todorov P, McDevitt TM, Cariuk P, Coles B, Deacon M, Tisdale MJ. Induction of muscle protein degradation and weight loss by a tumor product. *Cancer Res* 1996;56:1256–61.
- Orino E, Tanaka K, Tamura T, Sone S, Ogura T, Ichihara A. ATP-dependent reversible association of proteasomes with multiple protein components to form 26S complexes that degrade ubiquitinated proteins in human HL-60 cells. *FEBS Lett* 1991;284:206–10.
- Fenteany G, Schreiber SL. Lactacystin, proteasome function and cell fate. *J Biol Chem* 1998;273:8545–8.
- Andrews NC, Faller DV. A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. *Nucleic Acids Res* 1991;19:2499.
- Tanahashi N, Kawahara H, Murakami Y, Tanaka K. The proteasome-dependent proteolytic system. *Mol Biol Rep* 1999;26:3–9.
- Toker A. Signaling through protein kinase C. *Front Biosci* 1998;3:1134–47.
- Smith HJ, Tisdale MJ. Signal transduction pathways involved in proteolysis-inducing factor induced proteasome expression in murine myotubes. *Br J Cancer* 2003;89:1783–8.
- Nissen SL, Abumrad NN. Nutritional role of the leucine metabolite  $\beta$ -hydroxy  $\beta$ -methylbutyrate (HMB). *J Nutr Biochem* 1997;8:300–11.
- May PE, Barber A, D'Olimpio JT, Hourihane A, Abumrad NN. Reversal of cancer-related wasting using oral supplementation with a combination of  $\beta$ -hydroxy- $\beta$ -methylbutyrate, arginine and glutamine. *Am J Surg* 2002;183:471–9.
- Clark RH, Feleke G, Din M, et al. Nutritional treatment for acquired immunodeficiency virus-associated wasting using  $\beta$ -hydroxy  $\beta$ -methylbutyrate, glutamine, and arginine: a randomised, double-blind, placebo-controlled study. *J Parenter Enteral Nutr* 2000;24:133–9.
- Gomes-Marcondes MCC, Smith HJ, Cooper JC, Tisdale MJ. Development of an *in-vitro* model system to investigate the mechanism of muscle protein catabolism induced by proteolysis-inducing factor. *Br J Cancer* 2002;86:1628–33.
- Whitehouse AS, Tisdale MJ. Downregulation of ubiquitin-dependent proteolysis by eicosapentaenoic acid in acute starvation. *Biochem Biophys Res Commun* 2001;285:598–602.
- Fan X, Hunag X, DaSilva C, Castanga M. Arachidonic acid and related methyl ester mediate protein kinase C activation in intact platelets through the arachidonate metabolism pathways. *Biochem Biophys Res Commun* 1990;109:933–40.
- Vertegaal ACO, Kuiperij HB, Yamaoka S, Courtois G, Van der Eb AJ, Zantema A. Protein kinase C- $\alpha$  is an upstream activator of the I $\kappa$ B kinase complex in the TPA signal transduction pathway to NF- $\kappa$ B in U2OS cells. *Cell Sig* 2000;12:759–68.
- Chang L, Karin M. Mammalian MAP kinase signalling cascades. *Nature* 2001;410:37–40.
- Blumer KJ, Johnson GL. Diversity in function and regulation of MAP kinase cascade pathway. *Trends Biochem Sci* 1994;19:236–40.
- Burgering BMT, Bos JL. Regulation of Ras-mediated signalling: more than one way to skin a cat. *Trends Biochem Sci* 1995;20:18–22.
- Kolch W, Heidecker G, Kochs G, et al. Protein kinase C  $\alpha$  activates Raf-1 by direct phosphorylation. *Nature* 1993;364:249–52.

<sup>2</sup> Unpublished observation.