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β-hydroxy-β-methylbutyrate (HMB) stimulates myogenic cell proliferation, differentiation and survival via the MAPK/ERK and PI3K/Akt pathways

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ABSTRACT

β-hydroxy-β-methylbutyrate (HMB), a leucine catabolite, has been shown to prevent exercise-induced protein degradation and muscle damage. We hypothesized that HMB would directly regulate muscle-cell proliferation and differentiation and would attenuate apoptosis, the latter presumably underlying satellite-cell depletion during muscle degradation or atrophy. Adding various concentrations of HMB to serum-starved myoblasts induced cell proliferation and MyoD expression as well as the phosphorylation of MAPK/ERK. HMB induced differentiation-specific markers, increased IGF-I mRNA levels and accelerated cell fusion. Its inhibition of serum-starvation- or staurosporine-induced apoptosis was reflected by less apoptotic cells, reduced BAX expression and increased levels of Bcl-2 and Bcl-X. Annexin V staining and flow cytometry analysis showed reduced staurosporine-induced apoptosis in human myoblasts in response to HMB. HMB enhanced the association of the p85 subunit of PI3K with tyrosine-phosphorylated proteins. HMB elevated Akt phosphorylation on Thr308 and Ser473 and this was inhibited by Wortmannin, suggesting that HMB acts via Class I PI3K. Blocking of the PI3K/Akt pathway with specific inhibitors revealed its requirement in mediating the promotive effects of HMB on muscle cell differentiation and fusion. These direct effects of HMB on myoblast differentiation and survival resembling those of IGF-I, at least in culture, suggest its positive influence in preventing muscle wasting.

1. Introduction

Muscle growth in postnatal vertebrates is a highly regulated process in which muscle progenitor cells (satellite cells) proliferate and differentiate to form multinucleated myotubes which will mature into new muscle fibers. In adults, the normally quiescent satellite cells re-enter the myogenic program in response to regenerative cues such as injury or exercise. This myogenic program is governed, at least in part, by the muscle-specific basic helix–loop–helix (bHLH) family of transcription factors [1,2]. Upon satellite cell activation, the muscle-specific transcription factors are expressed in a sequential pattern, with Myf5 and MyoD being expressed in the proliferating progeny, followed by myogenin expression as the cells enter differentiation [3–5]. Several signaling pathways have been implicated in transducing growth factor effects on myoblast proliferation, differentiation and survival. The mitogen-activated protein kinase/extracellular signal-regulated protein kinase (MAPK/ERK) pathway has been reported to inducing cell proliferation [6,7]. Conversely, the phosphoinositide 3′-kinase (PI3K)/Akt pathway has been reported to be play a major role in inducing cell differentiation and hypertrophy [8,9]. In addition, Akt has been previously shown to be involved in cell survival via inhibition of pro-apoptotic proteins [10,11] and activation of anti-apoptotic proteins [12].

Although the number of satellite cells decreases to less than 5% in mature adult skeletal muscle, they are the primary mediators of muscle regeneration throughout life, implying that they can be continuously renewed while maintaining the ability to generate differentiated progeny and new myofibers [13–15]. Nevertheless, in pathological conditions such as muscular dystrophies and cachexia, or with age, there is a decline in the absolute number of satellite cells, leading to muscle atrophy as well as a tremendous loss of myofibrillar proteins from the skeletal muscle and decreased protein synthesis [16–18]. Several reports have demonstrated that apoptosis is one of the main reasons for satellite cell depletion in over exercised or aged animals after injury [19,20], or during degeneration-regeneration cycles in myopathies [21,22].

β-hydroxy β-methylbutyrate (HMB), a metabolite of the essential branched-chain amino acid leucine, is produced endogenously in small amounts and is one of the latest dietary supplements for the promotion of gains in strength and lean body mass when associated with resistance training [23–27]. Other studies have shown that HMB is more beneficial in untrained individuals than in trained athletes

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[28–31]. The efficacy of HMB has also been demonstrated in pathological conditions, where it has been reported to reduce muscle wasting associated with muscular dystrophies, trauma and cancer cachexia [30,32,33]. In the *mdx* mouse model of Duchenne muscular dystrophy, HMB alone or in combination with corticosteroids has been shown to improve muscle function and to decrease retroperitoneal fat-pad deposition [32]. Nevertheless, HMB's mode of action, in general and in muscle in particular, is still controversial. HMB may provide a source of cytosolic HMG-CoA for cholesterol synthesis, allowing a larger supply of cholesterol for cell membrane synthesis and therefore, maintenance of sarcolemmal integrity [34]. Other reports have shown that HMB reduces cancer-induced muscle weight loss via attenuation of the ubiquitin–proteasome proteolytic pathway [33,35,36], suggesting that HMB functions predominantly as an anti-catabolic, rather than anabolic compound. However, a recent study has shown that in cultured mouse cachectic myotubes, HMB stimulates protein synthesis via the mTOR pathway, in addition to attenuating the depression in protein synthesis induced by the proteolysis-inducing factor [37]. In other cell systems, HMB has been shown to have direct effects on cell proliferation and function [38,39]. Together, these reports led us to hypothesize that HMB, in addition to protecting the muscle from degradation, also has a direct promotive effect on myogenesis in adult muscle.

HMB has been shown to reduce muscle atrophy and increase muscle hypertrophy by inhibiting muscle degradation [27]. However, its direct effect on myogenic processes has never been studied. Here we examined the effect of HMB on proliferation, differentiation and fusion, as well as on the survival of muscle cell cultures derived from non-mammalian and mammalian species. We show that it induces myoblast proliferation and that the MAPK/ERK pathway is required

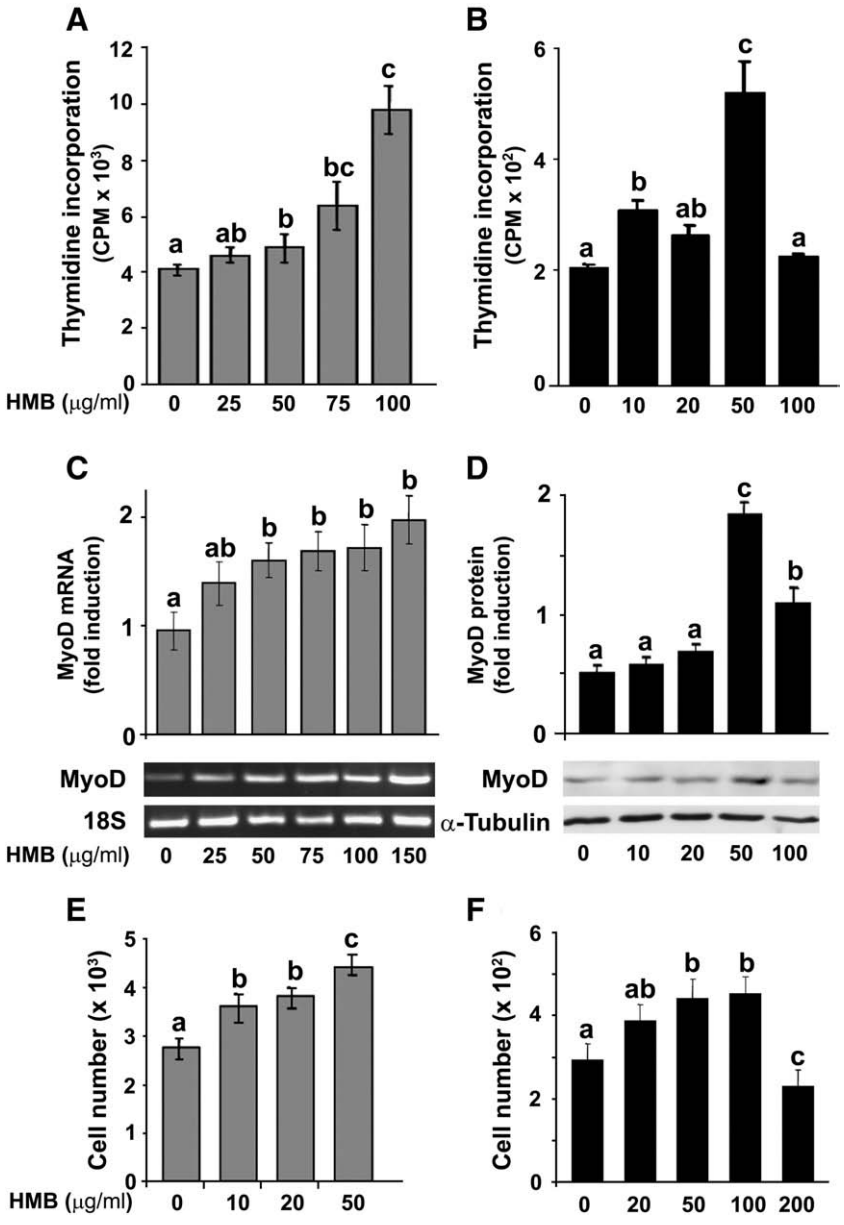

Fig. 1. Effect of HMB on labeled-thymidine incorporation into DNA in primary cultures of myoblasts derived from 3-day-old chick (A) or in human CHQ myoblasts (B). Cells were serum-starved for 40 h followed by addition of HMB at various concentrations for 17 h, after which [^{3}H]thymidine was added for an additional 4 h. Results are means ± s.e.m. of six replicates of three different experiments. (C) MyoD mRNA expression in response to various concentrations of HMB in chicken myoblasts. Cells were treated as in (A) and mRNA expression levels were evaluated by semi-quantitative RT-PCR analysis using 18S as an internal control (lower panel). (D) MyoD protein expression in response to HMB in CHQ cells. Densitometry analyses for MyoD levels normalized to 18S (C, upper panel) or to α-tubulin (D, upper panel) are presented as fold induction relative to control. Cell number in chicken (E) and human (F) cultures was determined by Coulter counter after 24-hour incubation. Values marked with different letters are significantly different ($P<0.05$).

for this effect. Moreover, HMB reduces muscle cell apoptosis triggered by cell starvation or staurosporine. The HMB-induced PI3K-dependent Akt phosphorylation is required for HMB's promotive effect on differentiation of myoblasts and myotube fusion. HMB triggers the synthesis of (insulin-like growth factor-I (IGF-I) mRNA, suggesting that the initial effects of HMB are boosted later on by IGF-I. Taken together, these data suggest that HMB has a beneficial effect on adult skeletal muscle, both during normal growth and under stress conditions.

2. Materials and methods

2.1. Materials

HMB was purchased from Metabolic Technologies Inc. Media and sera were obtained from Invitrogen and Biological Industries, respectively. Staurosporin was obtained from Sigma and UO126, Wortmannin and Ly294002 were purchased from Calbiochem.

2.2. Cell preparation and maintenance

Human myoblasts (satellite cells) were isolated as described previously [40] from the quadriceps muscle of a 5-d-old infant (termed here CHQ) in accordance with the French legislation on ethical rules and standards. Cells were grown in Ham's F10 medium supplemented with 20% (v/v) fetal bovine serum (FBS). Chicken myoblasts were isolated from the pectoralis muscle of 3-day-old chicks as previously described [41], under the approval of the Animal Welfare Committee of the Faculty of Agricultural, Food and Environmental Quality Sciences at the Hebrew University of Jerusalem. Cells were grown in DMEM containing 10% (v/v) horse serum. The mouse myogenic cell line C2 [42] was grown in 20% FBS-containing DMEM. All cells were maintained in 5% CO_2. For cell proliferation assays, cells were counted daily using a Coulter counter (Coulter Electronics).

2.3. Thymidine incorporation

DNA synthesis was assessed by [^{3}H]thymidine incorporation as previously described [41]. [^{3}H]thymidine (New England Nuclear) was added (2 μCi/well) for 4 h of incubation. Radioactivity in dissolved precipitates was counted using a Tri-Carb 1600CA scintillation counter (Packard). Equal plating efficiency was verified by determining cell numbers in parallel wells.

2.4. RNA preparation and RT-PCR

Total RNA was prepared using TRIzol™ Reagent (Invitrogen). Total RNA (1 μg) was reverse-transcribed into cDNA using random primers and SuperScript reverse transcriptase (Invitrogen). PCR was then performed using *Taq* DNA polymerase (Fermentas) for the following primers: 18S (F)—5′-CGA TGC TCT TAA CTG AGT GT-3′, 18S (R)—5′-TCA GCT TTG CAA CCA TAC TC-3′ (419 bp), MyoD (F)—5′-CGT GAG CAG GAG GAT GCA TA-3′, MyoD (R)—5′-GGG ACA TGT GGA GTT GTC TG-3′ (280 bp), IGF-I (F)—5′-GTA TGT GGA GAC AGA GGC TTC-3′, and IGF-I (R)—5′-TTT GGC ATA TCA GTG TGG CGC-3′ (200 bp). Thirty-five cycles (MyoD, IGF-I) or 20 cycles (18S) of amplification were performed, each consisting of 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C, followed by a final 10-minute extension at 72 °C. 18S was used as an internal control to normalize the sample mRNA amounts. PCR products were separated by electrophoresis and bands were visualized by video camera (Dinco-Ranium).

2.5. Immunoprecipitation and western blot analysis

Cells were extracted with lysis buffer as described in [43] and lysates were cleared by centrifugation. Equal amounts of protein were immunoprecipitated with monoclonal anti-phosphotyrosine antibody (anti-pTyr, Upstate Biotechnology). The immunopellets were resuspended in SDS-PAGE sample buffer under reducing conditions and subjected to electrophoresis and western blot analysis. Equal amounts of protein were loaded and resolved by 7.5% (w/v) SDS-PAGE, then transferred to nitrocellulose membranes (Biorad). After blocking, the membranes were incubated with the following primary antibodies: polyclonal anti-PI3K p85 pan (BD Biosciences), anti-phospho-MAPK/ERK, anti-MAPK/ERK, anti-phospho-Akt and anti-Akt (Cell Signaling), anti-MyoD, anti-MEF2 (Santa Cruz), monoclonal anti-myosin heavy chain (MHC; MF20) (Hybridoma Bank), anti-Bcl-2, anti-BAX (BD Pharmingen) and monoclonal anti-α-tubulin antibody (Oncogene).

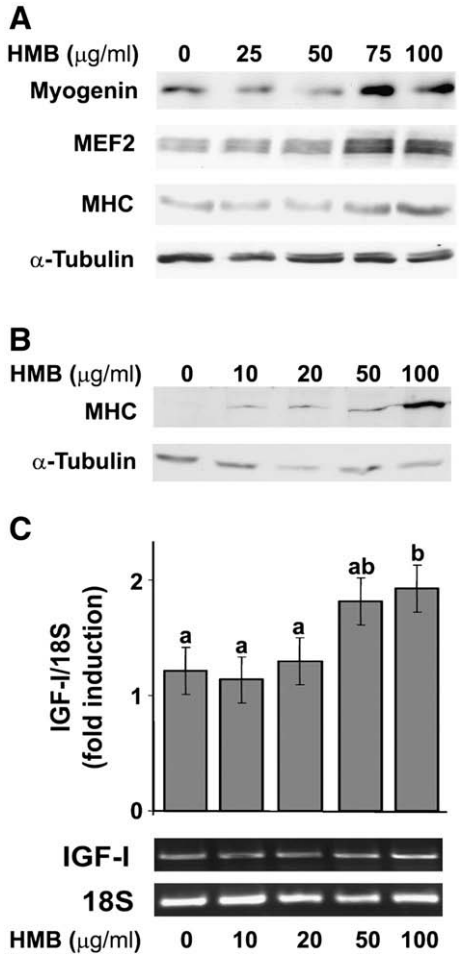

Fig. 2. HMB increases markers of myogenic differentiation. One-day primary cultures of chicken (A) or human (CHQ) myoblasts (B) were incubated in the absence or presence of HMB at various concentrations for 24 h, and protein expression levels of myogenin, MEF2 and MHC were analyzed by western blot. α-tubulin bands represent quantity of protein loaded. (C) Dose-dependent induction of IGF-I mRNA levels in response to HMB in chicken myoblasts. Cells were treated as in (A) and mRNA expression levels were evaluated by semi-quantitative RT-PCR analysis using 18S as an internal control (lower panel) followed by densitometry analysis for IGF-I normalized to 18S (upper panel).

2.6. Immunofluorescence analysis

Cells were fixed in 2% (w/v) paraformaldehyde for 15 min at room temperature. After blocking in 5% (v/v) goat serum, cells were incubated with the primary antibodies for 17 h at 4 °C, followed by washes in PBS and incubation with the secondary antibody for 30 min. The primary antibodies were: MF20 (1:10), rabbit anti-human Bcl-X (1:50) and rabbit anti-human Bcl-2 (1:200) (Epitomics). Secondary antibodies conjugated to either fluorescein-isothiocyanate (FITC) (Jackson Immuno Research) or Alexa-594 (Molecular Probes) were

used for labeling. Nuclei were counterstained with Hoechst 33258 (Sigma).

2.7. Fusion assays

Fusion was analyzed by nuclear number assays as described by Horsley et al. [44]. The number of nuclei in individual myotubes was counted for 600–700 myotubes and these were grouped into categories of either expressing two to four, or five or more nuclei. The percentage of myotubes in each category was calculated.

2.8. Flow cytometry analysis

Apoptosis was induced by adding 0.5 μM staurosporine (Sigma) to the proliferation medium for 16 h. Cells were collected, stained with Annexin-V-FITC (BD Pharmingen) and propidium iodide (PI, Molecular Probes), and analyzed by flow cytometry using a FacsCalibur and CellQuest software (Becton Dickinson). Percentage of apoptotic cells was defined by Annexin-V^+PI^- gated cells (adapted from Vermes et al. [45]). Annexin-V is a Ca^{2+}-dependent, phospholipid-binding protein that has a high affinity for phosphatidyl serine (PS) and binds to cells with exposed PS [45].

2.9. Statistical analysis

Data were subjected to one-way ANOVA and to Student's *t*-test or Tukey–Kramer test (for fusion analysis), using JMP® software [46].

3. Results

3.1. HMB promotes muscle cell proliferation

Chicken and human CHQ primary myoblast cell cultures, representing non-mammalian and mammalian species, were rendered quiescent by placing them in serum-free medium for 40 or 48 h for chick and human cells, respectively. We have previously shown that this type of starvation does not induce myogenic differentiation and that cells can be driven back into the cell cycle in the presence of mitogens [42,47]. HMB was then added at various concentrations for 17 h, followed by thymidine labeling for an additional 4 h. In both cell cultures, HMB increased the incorporation of thymidine into DNA by more than 2.5-fold relative to untreated control cells (Fig. 1A, B). Its effect was dose-dependent in chicken cells (Fig. 1A), whereas in the CHQ cells, a sharp peak was observed at 50 μg/ml HMB (Fig. 1B) ($P<0.05$). The mRNA level of the muscle regulatory factor, MyoD, which is upregulated in activated satellite cells, was elevated in response to the addition of HMB in a dose-dependent manner in chicken cells (Fig. 1C). In CHQ cells, the pattern of MyoD protein accumulation in response to HMB paralleled that of thymidine incorporation (Fig. 1D). Cell number increased dose-dependently in response to HMB in both cell cultures after 24 h of incubation (Fig. 1E, F). In CHQ cells, a significant reduction in cell number was observed at 200 μg/ml HMB (Fig. 1F).

The effect of HMB on cell proliferation was tested in myoblasts derived from 3-day-old chicks, in which proliferation activity is highest, and 7-day-old chicks when most of the proliferating myoblasts have undergone differentiation and myoblast proliferation and number have markedly declined [41]. As expected (Fig. 1A), in 3-day-old chicks, HMB affected thymidine incorporation in a dose–response manner. However, in 7-day-old chicks, cells were more sensitive to HMB and a maximal effect was observed at 5 μg/ml HMB (1000 ± 120 vs. 2600 ± 400 CPM for 0 and 5 μg/ml HMB, respectively, $P<0.05$). Thymidine incorporation declined at higher concentrations of HMB (data not shown).

3.2. HMB promotes cell differentiation and fusion

Addition of various concentrations of HMB to serum-starved chicken primary myoblasts for 24 h enhanced the protein levels of the muscle differentiation factors myogenin and MEF2 (Fig. 2A) in a dose-responsive manner, with the highest levels being observed at 100 μg/ml HMB. Similarly, MHC protein levels were induced in these cells as well as in CHQ myoblasts in response to HMB addition (Fig. 2A, B). The mRNA expression levels of IGF-I were dose-dependently induced by HMB up to nearly twofold compared to control untreated cells (Fig. 2C). HMB's potential role in the initial events of cell fusion was analyzed as early as 24 h of incubation by immunofluorescence analysis with an antibody against MHC in human CHQ cells, as well as in the mouse myogenic cell line, C2. In the case of C2, most of the control cells that expressed MHC were mononuclear (84.3 ± 0.03%, Fig. 3A). In the presence of higher concentrations of HMB, myotubes containing two and more nuclei were observed (Fig. 3B, C). Quantitation analysis of the number of nuclei in individual myotubes revealed a significant elevation in the percentage of myotubes containing two to four nuclei in response to HMB (11.6 ± 0.03%, 33.4 ± 0.02%, 30.6 ± 0.01% for 0, 20, 50 μg/ml HMB, respectively, $P<0.05$). The number of myotubes containing five or more nuclei was highest in the presence of 50 μg/ml HMB (6.1 ± 0.02% vs. 38.4 ± 0.03% for 20 and 50 μg/ml HMB, respectively, $P<0.05$).

Control CHQ cells expressed MHC but remained small and mononuclear (96.7 ± 0.03%, Fig. 3D), while in the presence of 100 μg/ml HMB (Fig. 3E) or 7.5 ng/ml IGF-I (Fig. 3F), larger MHC-

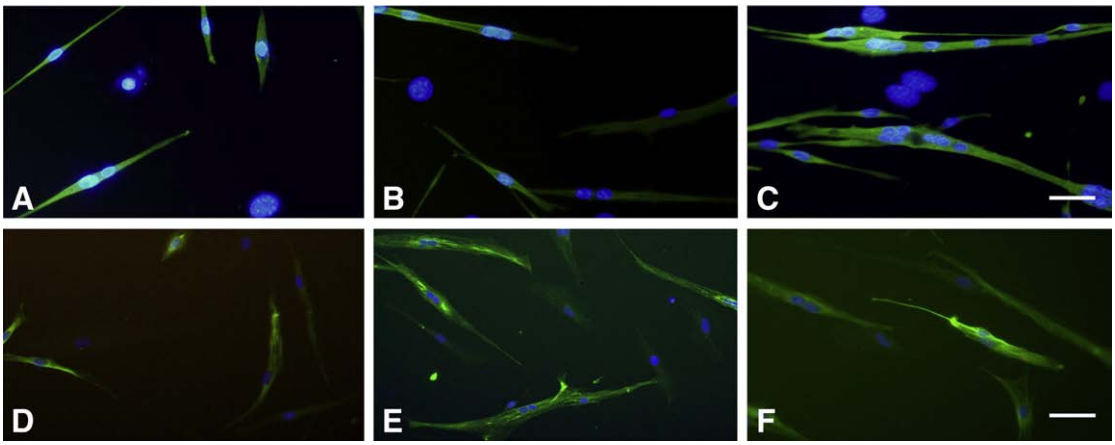

Fig. 3. Immunofluorescence staining for MHC expression in muscle cells treated with HMB. Mouse C2 cells (A–C) and human CHQ cells (D–F) were treated as described in Fig. 2 with or without various concentrations of HMB or with IGF-I and immunostained for MHC. Control, untreated cells (A, D), 20 μg/ml HMB (B), 50 μg/ml HMB (C), 100 μg/ml HMB (E) and 7.5 ng/ml IGF-I (F). Bar, 20 μM for A, B, C, 40 μM for D, E, F.

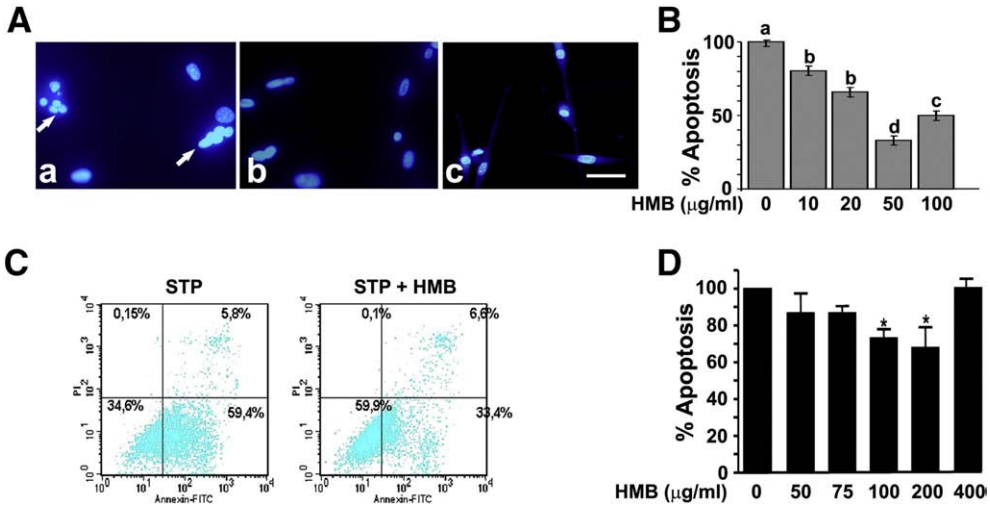

Fig. 4. HMB reduces muscle cell apoptosis. (A) C2 cells were serum-starved for 48 h, after which DMEM was replaced with fresh medium in absence (a) or presence of 20 μg/ml (b) and 50 μg/ml (c) HMB for an additional 24 h. DNA nuclei were stained with Hoechst dye. Note the fragmented/apoptotic nuclei (arrows) vs. the intact ones in control and HMB-treated cells, respectively. (B) Apoptotic nuclei were counted and data are presented as a percentage of total nuclei. Values are expressed as means ± s.e.m. of three independent experiments in which more than 700 cells were examined. Values marked with different letters are significantly different ($P<0.05$). (C) Human CHQ myoblasts were induced to apoptosis with staurosporine (STP; 0.5 μM) in the absence or presence of HMB. After 6 h cells were stained with FITC-conjugated Annexin-V and PI. Percentage of Annexin-V/PI-stained cells was determined by flow cytometry and this figure represents one of the HMB concentrations. The dot plot was divided into quadrants: necrotic cells (upper left), early necrotic cells (upper right), living cells (lower left), and apoptotic cells (lower right). Graph demonstrates the number of apoptotic cells (lower right) in each treatment compared to that of control cells treated with staurosporine alone (D). Values are expressed as means ± s.e.m. of three independent experiments. *$P<0.05$ vs. control, untreated cells.

expressing cells were observed, some of which fused into myotubes containing two or more nuclei (2.9 + 0.01%, 9.9 + 0.01%, 10.4 + 0.02% for 0, 100 μg/ml HMB and IGF-I, respectively, $P<0.05$).

3.3. Effects of HMB on cell survival

One of the ways in which HMB may improve muscle growth and more importantly, prevent muscle atrophy, is by enhancing muscle cell survival and inhibiting apoptosis. To address this hypothesis, apoptosis was induced by means of serum starvation or staurosporine addition in C2 and CHQ cells, respectively. Previous studies have shown the apoptotic effect of serum starvation in myoblasts [48,49]. C2 cells were serum-deprived for 48 h, after which HMB was or was not added to the cells for an additional 24 h under the same conditions. Cytochemical labeling of nuclei with Hoechst dye revealed that HMB prevents the appearance of pycnotic nuclei (apoptotic nuclei), the latter being observable only in the control serum-starved cells (Fig. 4A). Quantitation analysis revealed that the percentage of apoptosis, represented as the percent of pycnotic nuclei out of total nuclei (20.3 ± 0.02% in control cells), was decreased by HMB in a dose-responsive manner being almost threefold lower than controls at 50 μg/ml (Fig. 4B). Human CHQ cells were grown for 1 day in medium containing 20% FBS after which HMB was added at various concentrations for 10 h followed by the addition staurosporine (0.5 μM) to induce apoptosis. Cells without the addition of staurosporine served as controls. Cell death or apoptosis was evaluated after 6-hour incubation by flow-cytometry analysis using PI staining and an antibody to Annexin-V. Annexin-V conjugated to fluorescein isothiocyanate (FITC) fluorochrome retains its high affinity for PS and can therefore serve as a sensitive probe for the flow-cytometric detection of cell death. A typical flow-cytometric plot of CHQ cells labeled with Annexin and PI is shown in Fig. 4C. Four different subpopulations of cells were detected: non-fluorescent viable cells (Fig. 4C, lower left panels), apoptotic cells labeled with Annexin-FITC, but not with PI (Fig. 4C, lower right panels), early necrotic cells labeled with Annexin-FITC and PI (Fig. 4C, upper right panels), and cells labeled with PI but not with Annexin-FITC, representing fully necrotic cells (Fig. 4C, upper left panels). In Fig.

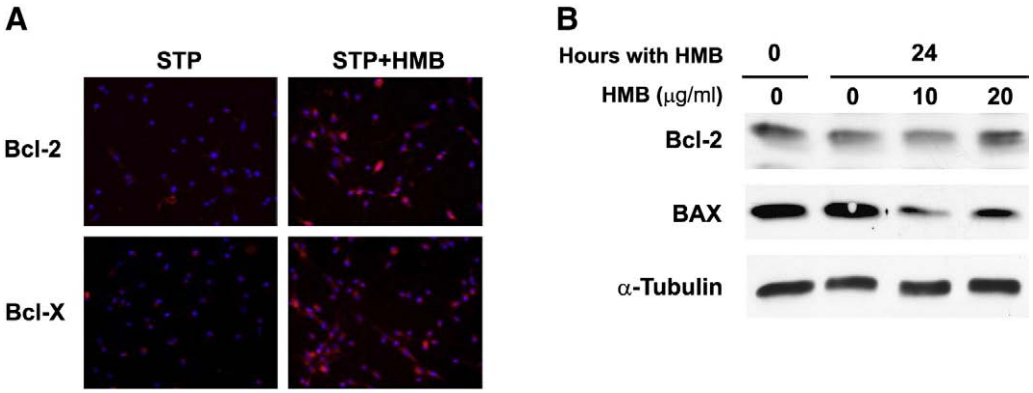

Fig. 5. Expression levels of pro- and anti-apoptotic proteins are affected by HMB in muscle cells. Apoptosis was induced in CHQ myoblasts by staurosporine (STP) as described in Fig. 4 and cells were treated with or without HMB (200 μg/ml). Bcl-2 and Bcl-X were analyzed by immunofluorescence analysis and nuclei were stained with DAPI (A). (B) Western blot analysis for Bcl-2 and BAX proteins in serum-starved C2 cells. Cells were serum-starved for 48 h (zero time) then treated with 10 or 20 μg/ml HMB for an additional 24 h (24 h time). α-tubulin bands represent quantity of protein loaded.

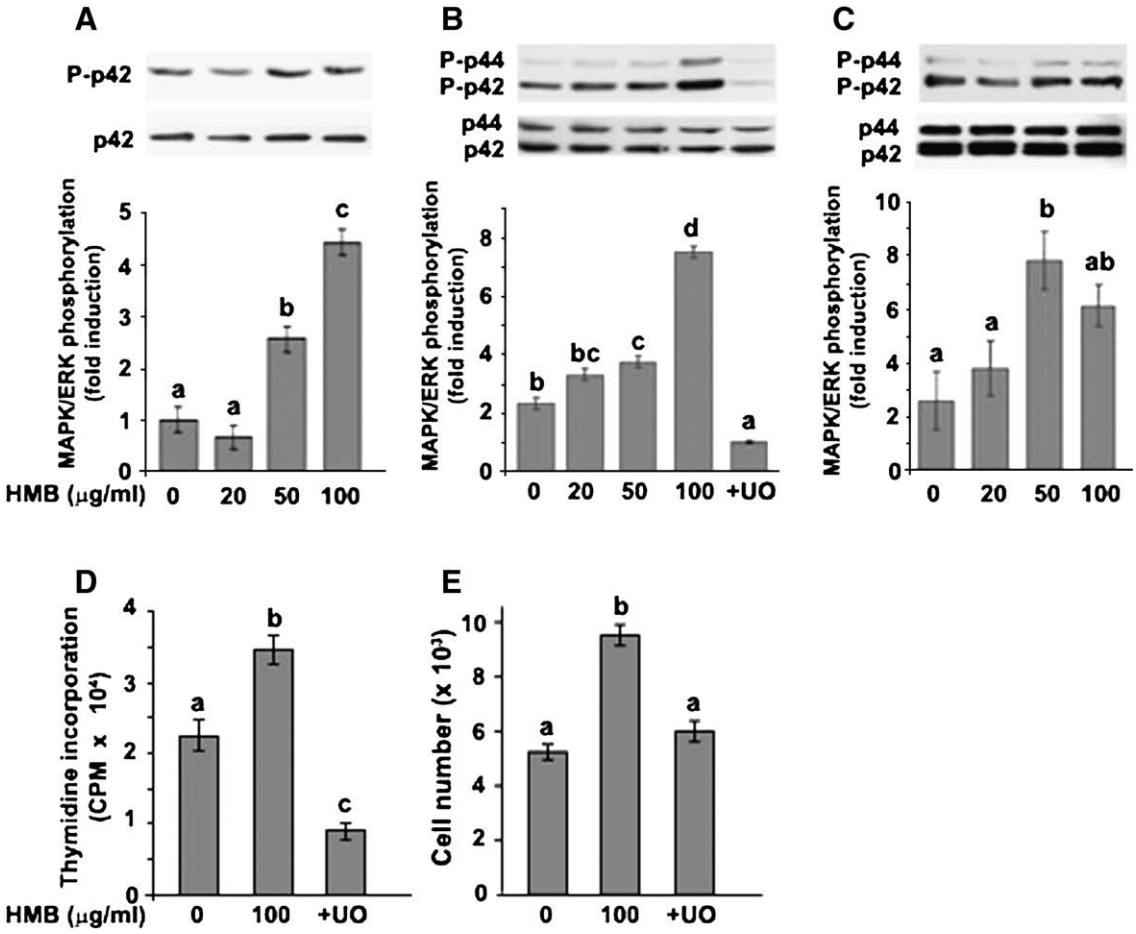

Fig. 6. The MAPK/ERK pathway mediates the HMB promoting effect on muscle cell proliferation. Serum-starved C2 cells (A) or CHQ myoblasts (B) were stimulated with various concentrations of HMB for 12 min. Cell lysates were resolved by SDS-PAGE and subjected to immunoblotting with anti-phospho-ERK1/2 antibody. The specificity of this induction is demonstrated in C2 cells by UO126 (UO, 10 μM) treatment 30 min prior to HMB (20 μg/ml) addition. Parallel membranes were probed with anti-total ERK1/2 and anti-total Akt antibodies. Upper panels: representative western blot analysis of MAPK/ERK phosphorylation. Lower panels: bands were quantified by densitometry relative to total MAPK/ERK and results are means ± s.e.m. of three independent experiments. Data with different letters differ significantly ($P<0.05$). Thymidine incorporation into DNA (D) and cell numbers (E) of C2 cells were tested in the presence of 100 μg/ml HMB without or with 10 μM UO126 (+UO). Cells were treated as described in Fig. 1. Results are means ± s.e.m. of six replicates of three different experiments. Data with different letters differ significantly ($P<0.001$).

4D, the percentage of apoptotic CHQ cells treated with increasing concentrations of HMB was normalized to that of cells treated with staurosporine alone (0) representing 100% (calculations included cells that were found in the lower right panels). The percentage of apoptosis was reduced by HMB in a dose-dependent manner, by more than 30% at 200 μg/ml HMB; however, higher concentrations of this compound reversed the effect, with the percentage returning to control levels.

Next, we evaluated the expression levels of the Bcl-family members which play a role in the immediate events of the apoptotic response. CHQ cells were either treated or not treated with 200 μg/ml HMB for 24 h, after which the medium was replaced with fresh medium containing HMB (200 μg/ml) in a similar manner for an additional 4 h, followed by the addition of staurosporine (0.5 μM) to all plates to induce apoptosis. After 4 h of incubation with staurosporine, cells were subjected to immunofluorescence analysis for Bcl-2 and Bcl-X. Both proteins were widely expressed in cells incubated in the presence of staurosporine and HMB, whereas their expression was low in the presence of staurosporine alone (Fig. 5A). Quantitative analysis of two independent experiments revealed that the percentage of cells expressing Bcl-2 or Bcl-X out of the total number of cells increased from 22 to 49% and from 42.9 to 77.9%, respectively, in the presence of HMB. A similar trend was observed in serum-starved C2 cells: whereas Bcl-2 protein levels declined in the untreated cells from 48 to 72 h of serum deprivation, 20 μg/ml HMB prevented this decline (Fig. 5B, compare time zero and 24 h). A lower level of HMB had no effect on Bcl-2 levels. Conversely, the level of BAX, an apoptosis-promoting molecule, was lower in the HMB-treated cells compared to controls (Fig. 5B).

3.4. The MAPK/ERK and PI3K/Akt signaling pathways are induced by HMB

In view of our results, we sought to determine whether HMB induces signaling pathways such as those of MAPK/ERK and PI3K/Akt. Serum-starved cells were incubated with various concentrations of HMB for 12 min followed by western blot analysis for phosphorylated and total MAPK/ERK proteins. MAPK/ERK phosphorylation was dose-dependently induced in the chicken (Fig. 6A) and C2 cells (Fig. 6B) and this phosphorylation was abolished in the presence of a MEK-specific inhibitor UO126 (Fig. 6B). Similarly in CHQ cells, MAPK/ERK phosphorylation was induced by increasing concentrations of HMB, peaking at 50 μg/ml (Fig. 6C). To test whether the ERK/MAPK pathway mediates HMB's effects on muscle cell proliferation, serum-starved C2 cell were treated with 100 μg/ml HMB with or without UO126 that was added 30 min prior to HMB addition. Thymidine incorporation into DNA (Fig. 6D) and number of cells (Fig. 6E) were evaluated after 17 and 24 h, respectively. In both cases, addition of UO126 to HMB-treated cells abolished the promoting effect of HMB on cell proliferation.

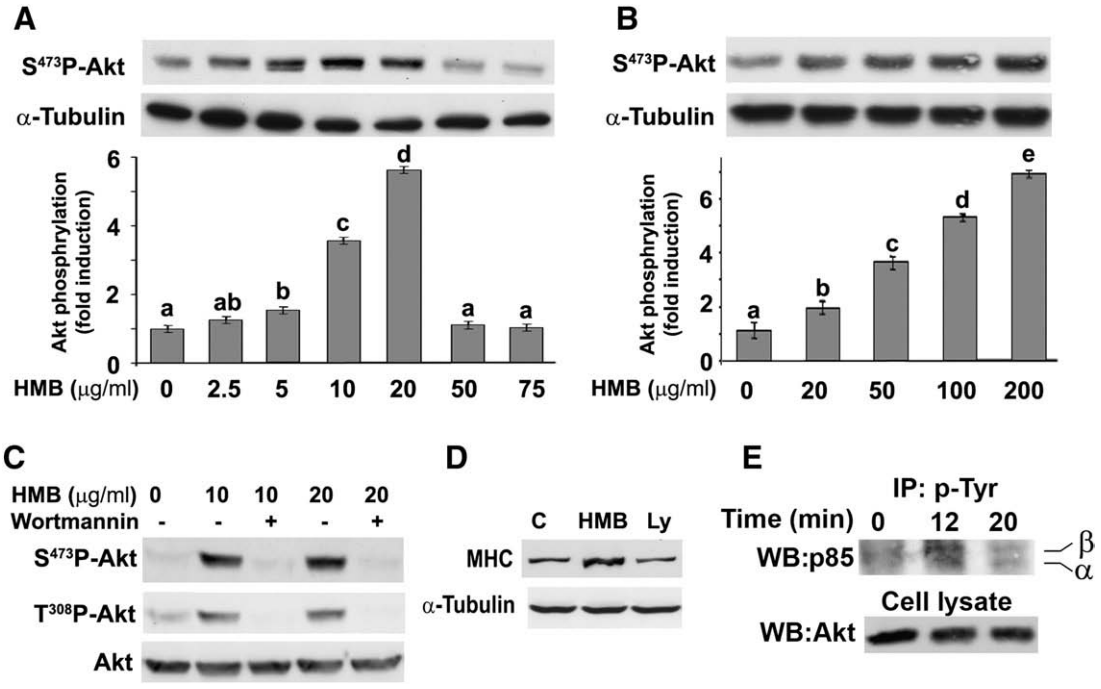

Fig. 7. The PI3K/Akt pathway is induced by HMB. Akt phosphorylation of serum-starved C2 (A) and human myoblasts (B) in response to various concentrations of HMB for 12 min. Akt phosphorylation was analyzed by western blot with an anti-phospho-Akt (Ser473) (S^{473}P-Akt) antibody followed by densitometric analysis normalized to levels of α-tubulin. Data with different letters differ significantly ($P<0.05$). (C) Analysis of Akt phosphorylation at amino acid residues Thr308 (T^{308}P-Akt) and Ser473 in C2 cells in the presence of various concentrations of HMB, with or without Wortmannin (100 nM) for 12 min. (D) A western blot analysis for MHC in C2 cells treated for 24 h with 100 μg/ml HMB without (HMB) or with Ly294002 (25 μM, Ly). C, control non-treated cells. (E) Recruitment of p85α and p85β isoforms of PI3K to tyrosine-phosphorylated proteins (p-Tyr) in response to HMB (20 μg/ml). Serum-starved C2 cells were harvested with lysis buffer and subjected to immunoprecipitation (IP) with anti-pTyr antibody. Immunoprecipitates were resolved by 7.5% SDS-PAGE and subjected to immunoblotting with anti-p85 pan antibody. Protein quantity is represented by western blot for total Akt in cell lysates.

In serum-starved C2 cells, Akt phosphorylation on amino acid Ser473 was upregulated after 12 min of incubation with HMB in a bell-shaped manner, to approximately sixfold more than control levels at 20 μg/ml HMB (Fig. 7A). Akt phosphorylation was induced in CHQ cells in a dose-dependent manner (Fig. 7B). HMB induced the phosphorylation of Akt phosphorylation sites, Ser473 and Thr308, both of which are required for full kinase activity (Fig. 7C) [50,51]. This phosphorylation was completely abolished in the presence of the specific PI3K inhibitor Wortmannin (100 ng/ml). To test whether Akt phosphorylation is required for HMB's stimulatory effect on myoblast differentiation and fusion initiation, C2 myoblasts or myotubes were treated with 50 μg/ml HMB for 24 h with or without Ly294002, a stable PI3K inhibitor, which was added 30 min prior to the HMB addition. A western blot analysis for MHC revealed that Ly294002 addition prevented the HMB-induced MHC levels in C2 cells (Fig. 7D). Quantitation analysis of the number of nuclei in individual myotubes revealed that Ly29004 treatment prevents the elevation in the percentage of myotubes containing two to four nuclei in response to HMB ($30.4\pm0.17\%$ and $1.7\pm0.01\%$ for 50 μg/ml HMB alone or with Ly294002, respectively, $P<0.05$), and more than 95% of the cells remained in their mononuclear state.

To analyze whether HMB is involved in the activation of Class I PI3K, C2 cells were treated with or without 20 μg/ml HMB for various times and cell lysates were subjected to immunoprecipitation with anti-pTyr antibody. Western blot analysis with anti-p85 pan antibody, which reacts with all isoforms of endogenous p85, revealed that HMB induces the association of both p85α and p85β isoforms with tyrosine-phosphorylated proteins (Fig. 7E).

4. Discussion

HMB has been suggested to influence muscle strength via various mechanisms, such as decreasing protein degradation [27] and attenuating the proteosome–ubiquitin degradation pathway [35,36] on the one hand, and increasing protein synthesis [37] on the other. In this study, we provide the first report of HMB's positive influence on muscle via the promotion of proliferation and differentiation, the acceleration of fusion, and the reduction of apoptosis in adult myoblast cultures.

Direct addition of HMB to primary cultures of chicken and human muscle cells, revealed its ability to stimulate cell proliferation. In both species, the expression levels of MyoD, a marker for activated satellite cells [3–5], thymidine incorporation by DNA and cell numbers were increased in response to HMB. This is in complete agreement with previous reports describing the mitogenic effect of HMB in other cell types [38,39]. Although our cells were derived from young chick and human skeletal muscles, they were rendered quiescent in order to mimic their state in adult skeletal muscle. Moreover, following HMB treatment, we observed enhanced proliferation of chicken myoblasts derived from older chicks, in which satellite cell activity was very low [41,52]. Taken together, our findings suggest that, at least in culture, HMB has the ability to drive quiescent adult myoblasts into the cell cycle, thus acting as a mitogen in these cells, regardless of the animal's age. The differential response of human and chicken cells to HMB suggests this mitogen's species-specific effect.

In addition to its mitogenic effect, HMB promoted terminal muscle-cell differentiation and fusion. HMB increased in a dose-dependant manner the protein levels of myogenic regulatory factors, MEF2 as well as of MHC, and accelerated the appearance of myotubes. This was evident in myoblasts derived from both chick and human primary cell cultures, as well as in the C2 mouse cell line. HMB's effect on muscle cell differentiation has also been observed in in-vivo studies: administration of HMB to chick embryos by in-ovo feeding enhances the expression levels of myogenic regulatory factors in muscles of posthatch chicks relative to untreated controls (R. Kornasio, Z. Uni and O. Halevy, unpublished). The fact that HMB

triggered increased expression of IGF-I mRNA in primary chick myoblasts raises the possibility of its affecting myoblast differentiation via locally expressed IGF-I induction; this would induce the expression of interleukin-4 and interleukin-13 [53], thereby promoting cell fusion. This muscle-produced isoform of IGF-I is known for its induction of muscle cell differentiation and hypertrophy [54].

The finding that proliferation and differentiation are induced by 24-hour treatment with HMB has been reported for other factors, such as Shh [47]. Our explanation for this phenomenon is that the 2-day starvation of myoblasts causes their withdrawal from the cell cycle, but in a reversible manner. At the same time, this starvation period renders some of the cells more prone to differentiation cues (perhaps the cells that initiated this process beforehand). This implies an heterogeneous population of muscle cells, in particular in the case of the primary ones. Previously, we reported that primary myoblasts derived from chicks at various ages, differ in their capacity to proliferate or differentiate [52,55] Because HMB stimulates both processes, some cells will proliferate better, whereas others will start to differentiate in response to this amino acid.

Apoptosis is considered to be one of the mechanisms involved in satellite cell depletion in cases such as trauma and aging, or during degeneration–regeneration cycles in myopathies [19–22], thereby contributing to an impaired skeletal muscle regeneration response. In this study, we demonstrate that HMB reduces staurosporine- or starvation-induced apoptosis in cultured human myoblasts and mouse C2 cells, respectively. The number of apoptotic cells was reduced in the presence of HMB and these cells exhibited higher levels of the anti-apoptotic proteins Bcl-2 and Bcl-X and lower levels of the pro-apoptotic protein BAX. These proteins, all of which belong to the Bcl-2 family, regulate apoptosis by controlling mitochondrial integrity: the anti-apoptotic proteins sequester the pro-apoptotic ones, thereby preventing permeabilization of the outer mitochondrial membrane and subsequent activation of downstream caspases [56]. Taken together, the beneficial effect of HMB on muscle strength and function in cases of muscle injury or wastage in trauma or cachexia [32–36,57] might be explained in part by its protection of satellite cells from depletion by apoptosis.

The signaling pathways via which HMB, a leucine catabolite, exerts its effects are not fully understood. In this study, we demonstrate for the first time the promotive effect of HMB on MAPK/ERK and Akt phosphorylation and the requirement of these pathways to mediate its effects, as demonstrated by employing specific inhibitors. Our results suggest that the MAPK/ERK pathway mediates HMB's effects on myoblast proliferation (Fig. 6D, E), in agreement with earlier reports [6,7,47]. By blocking the PI3K/Akt pathway with specific inhibitors, we demonstrate that this pathway is required in mediating the promotive effects of HMB on both, muscle cell differentiation and fusion acceleration. The crucial role of Akt in myogenic differentiation and hypertrophy has been previously demonstrated [8,9]. Moreover, Akt has been shown to phosphorylate pro-apoptotic proteins and to activate Bcl-2 expression [10–12], thus preventing apoptosis.

Recently, HMB has been reported to induce protein synthesis in C2C12 myotubes via the mTOR/p70S6k pathway [37], a feature that is shared with leucine [58]. Recent studies have reported that a class III PI3K, human vacuolar protein sorting-34 (hVps34), mediates the amino acid activation of mammalian target of rapamycin (mTOR) and its downstream molecules, which is distinct from the effect of insulin, which acts through class I PI3K [59,60]; these findings led Drummond and Rusmussen [61] to conclude that leucine acts via the class III and not class I PI3K pathway. Yet, one study has demonstrated that leucine phosphorylates class I PI3K although no effects on its downstream target, Akt were observed [62]. Our findings indicate that HMB transduces its signal via class I PI3K pathway: HMB enhances the recruitment of p85, the regulatory subunit of class I PI3K, to tyrosine-phosphorylated proteins and fully activates Akt by inducing phosphorylation on both, Thr307 and Ser473 [50,51]; this latter phosphorylation is inhibited by Wortmannin, a specific PI3K inhibitor. This distinction may give an additional explanation to the suggestion of HMB being more potent than leucine in preventing muscle degradation [35–37]. We cannot rule out the possibility that HMB transduces its signal also via hVps34 and activates the mTOR/p70S6k pathway in a manner similar to other amino acids. However, due to the notion that other amino acids signal directly to mTOR independent of the Akt signaling pathway [59–62], we believe that HMB acts predominantly via the class I PI3K/Akt pathway rather than the class III PI3K pathway.

The effects of HMB, at least in culture, on myoblast proliferation and differentiation, as well as on apoptosis in adults, resemble those of IGF-I. IGF-I has been reported to prevent muscle atrophy and wastage [63], ameliorate muscle mass and force generation, and reduce contraction-induced damage in muscles of dystrophic *mdx* mice [64,65]. Although it is conceivable that HMB affects differentiation via IGF-I, its effect is more likely to be direct. First, HMB's effect on cell morphology and fusion was similar to that of IGF-I at the same time point (Fig. 3F). Second, it had an immediate effect on Akt phosphorylation (Fig. 7A, B) and on recruitment of p85 (Fig. 7E) but not insulin receptor substrate-1 (IRS-1, data not shown) to Tyr-phosphorylated proteins, suggesting the recruitment PI3K to some other tyrosine-phosphorylated signaling complex. Because HMB elevated IGF-I mRNA levels, we suggest that HMB has an immediate direct effect on muscle cell differentiation and survival and that these effect may be boosted later on via IGF-I secretion from these cells. Our findings show a bell-shaped pattern for HMB's effects in all muscle cells and in particular in human myoblasts. This resembles the activity pattern of other growth factors, due to down-regulation and internalization of their receptors. Although we do not know of any specific receptor for HMB, we speculate that the bell-shaped activity pattern indicates the existence of one.

In myopathies, the leading cause of satellite cell apoptosis as well as muscle deterioration is continuous degeneration–regeneration cycles in the damaged muscles [22,23]. Therefore, we believe that the promotive effects shown here of HMB on muscle cell proliferation and fusion, as well as on cell survival, together with its previously demonstrated anti-catabolic effects [33,35,36], justify this supplement as a therapeutic strategy to prevent muscle loss in myopathies as well as in aging, trauma and cancer cachexia.

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