Attenuation of depression of muscle protein synthesis induced by lipopolysaccharide, tumor necrosis factor, and angiotensin II by β-hydroxy-β-methylbutyrate

Helen L. Eley, Steven T. Russell, and Michael J. Tisdale
Nutritional Biomedicine, School of Life and Health Sciences, Aston University, Birmingham, United Kingdom

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The current study investigates the ability of HMB to attenuate the depression of protein synthesis in murine myotubes in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

ATROPHY OF SKELETAL MUSCLE IS COMMON in a number of conditions, including cancer (13), sepsis (27), human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS) (14), congestive heart failure (CHF) (3), and diabetes (20), leading to an increased morbidity and mortality. In all cases, atrophy results from an imbalance between the rates of protein synthesis and degradation, and in general, increased rates of protein degradation are combined with a depressed rate of protein synthesis.

Although the mechanism for the increased rate of protein degradation appears to be due to an increased expression and activity of the ubiquitin-proteasome proteolytic pathway (17), there is less information on the mechanism for the depression of protein synthesis. We (11) have recently shown that for proteolysis-inducing factor (PIF) and angiotensin II (ANG II), the efficiency of protein synthesis is reduced at the initiation phase of translation, through phosphorylation of eukaryotic initiation factor 2 (eIF2) on the α-subunit, as a result of activation of the double-stranded RNA-dependent protein kinase (PKR). In this step, initiator methionyl tRNA binds to the 40S ribosomal subunit as a tertiary complex with eIF2 and GTP. eIF2 is released in its GDP-bound state, and to return to its active GTP-bound form, the GDP is exchanged for GTP by the guanine nucleotide exchange factor eIF2-B (22). Phosphorylation of eIF2 on the α-subunit inhibits this exchange reaction, depressing the rate of protein synthesis (24).

A second control point in translation recruits the 40S ribosomal subunit to mRNA through the eIF4F triad of translation initiation factors. The eIF4F complex is composed of three subunits: eIF4E, eIF4A, and eIF4G, of which eIF4E is one of the main regulatory initiation factors and is present in low molar amounts in the cell. The concentration of eIF4E is also regulated by its association with its binding protein, 4E-BP1. Hyperphosphorylation of 4E-BP1 results in the release of eIF4E from the inactive eIF4E-4E-BP1 complex, allowing it to associate with eIF4G to form the active eIF4F complex (23). This process is initiated by branched-chain amino acids, such as leucine (4), whereas in the skeletal muscle of cachetic mice has been shown to be attenuated by β-hydroxy-β-methylbutyrate (HMB) (25), a metabolite of leucine, which has been shown to increase lean body mass in patients with cancer cachexia (19). In murine myotubes, HMB attenuated autophosphorylation of PKR and the subsequent phosphorylation of eIF2α in response to PIF, increased phosphorylation of 4E-BP1, releasing eIF4E to form the active eIF4E-eIF4G complex, and reduced phosphorylation of eIF2 (9). These results provide a mechanism by which HMB attenuates the depression of protein synthesis in cancer cachexia and suggest that it also may be effective in other conditions of muscle atrophy.

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response to ANG II, lipopolysaccharide (LPS), and tumor necrosis factor-α (TNF-α) as models of muscle atrophy in CHF (2), sepsis, and HIV/AIDS.

MATERIALS AND METHODS

Materials. Fetal calf serum (FCS), horse serum (HS), and Dulbecco’s modified Eagle’s medium (DMEM) were purchased from Invitrogen.
Paisley, UK). L-[2,6-3H]phenylalanine (specific activity 60 Ci/mmol) was obtained from ARC (Cardiff, UK). Hybond A nitrocellulose membranes, m7GTP Sepharose 4B, and enhanced chemiluminescence (ECL) development kits were obtained from Amersham Biosciences (Little Chalfont, UK). Rabbit polyclonal antisera to total and phospho-EF2 (eEF2 Thr56), total and phospho-4E-BP1 (Thr37/46), total and phospho-eIF4E (Ser209), eIF4G, total and phospho-PKR (Thr446), and total and phospho-target of rapamycin (mTOR) (Ser2448) were purchased from New England Biolabs (Hitchin, UK). Rabbit polyclonal antisera to total eIF2 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and that to phospho-eIF2 (Ser51), together with Phosphosafe extraction reagent, were obtained from Merck Eurolab (Lutterworth, UK). Peroxidase-conjugated goat anti-rabbit antibody was purchased from Dako (Cambridge, UK). ANG II, LPS from Escherichia coli 0111:B4, TNF-α, and IFN-γ were purchased from Sigma-Aldrich (Dorset, UK). The C2C12 myoblasts were obtained from the European Collection of Cell Cultures (Salisbury, UK).

Myogenic cell culture. C2C12 murine myoblasts were routinely propagated in DMEM supplemented with 10% FCS, glutamine, and 1% penicillin-streptomycin under an atmosphere of 10% CO2 in air. When the myoblasts reached about 80% confluence, they were differentiated into myotubes by replacing the growth medium with DMEM containing 2% HS, with the medium being changed every 2 days. Differentiation occurred within 3–5 days.

Measurement of protein synthesis in myotubes. The method for the determination of protein synthesis in murine myotubes has been described previously (13). All experiments were carried out in six-well multiwell dishes. Protein synthesis was determined from the incorporation of 370 kBq of L-[2,6-3H]phenylalanine into acid-insoluble material over the time periods specified, in the presence or absence of ANG II, LPS, or TNF-α/IFN-γ at the concentrations indicated. HMB (50 μM) was added 2 h before the stimulus.

Western blot analysis. Myotubes were treated with the various agents as indicated, scraped off the plates, and washed with PBS, followed by lysis in Phosphosafe extraction reagent for 5 min at room temperature and sonication at 4°C. The lysate was cleared by centrifugation at 18,000 g for 5 min at 4°C, and samples of cytosolic protein (5–15 μg) were resolved on 10% SDS-PAGE at 180 V for 1 h, followed by transference to 0.45-μm nitrocellulose membranes, which were then blocked with 5% Marvel in Tris-buffered saline, pH 7.5, at 4°C overnight. For eIF2α, 6% SDS-PAGE was used. The primary antibodies for PKR, eIF2α, mTOR, and eEF2 were used at a dilution of 1:1,000, and the secondary antibody was also used at a dilution of 1:1,000. Incubation was for 1 h at room temperature or overnight, and development was by ECL. Blots were scanned by a densitometer to quantify differences. To determine the association of eIF4E with 4E-BP1 and eIF4G, myotubes were 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 supernatant formed by centrifugation at 15,000 g for 15 min was added to a microcentrifuge tube containing 80 μl of m7GTP-Sepharose 4B at 4°C for 1 h. After centrifugation at 13,000 g and three washes in 1 ml of buffer A, the slurry was resuspended in 80

Fig. 3. Effect of ANG II (0.5 μM; A), TNF-α (50 ng/ml; B), and LPS (10 ng/ml; C) on phosphorylation of mammalian target of rapamycin (mTOR) at Ser2448 in murine myotubes in the presence of HMB (50 μM) for 4 h (A), 2 h (B), and 4 h (C). The total forms acted as loading controls. Representative Western blots are shown, and histograms represent the averages of 3 separate blots. D: effect of rapamycin (Rap) (10 ng/ml) on protein synthesis in murine myotubes in the presence of LPS (10 ng/ml for 4 h), TNF-α (50 ng/ml for 2 h), and ANG II (0.5 μM for 4 h) alone or with HMB (50 μM). *P < 0.001 compared with control. †P < 0.001 in the presence of HMB. ‡P < 0.001 in the presence of Rap.
ml of 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 quantitated by Western blotting, as described above. The antibodies were used at a dilution of 1:1,000, except for phospho-4E-BP1 (1:500). The amount of eIF4G and 4E-BP1 bound to m⁷GTP-Sepharose represents the association with eIF4E.

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

**RESULTS**

The effect of HMB on protein synthesis after 24 h of incubation with LPS is shown in Fig. 1A. LPS inhibited protein synthesis by 40–50% at concentrations between 1 and 100 ng/ml, and this effect was completely attenuated by HMB (50 μM). TNF-α, alone and in combination with IFN-γ, inhibited protein synthesis by 30 and 50%, respectively, at 2 h after addition, and this effect also was attenuated by HMB (Fig. 1B). There was no significant effect of TNF-α on protein synthesis at any other time point examined (30 min, 4 h, 8 h, 24 h). ANG II produced a depression of protein synthesis between 60 and 70% after 4 h of administration, and this was completely attenuated by HMB at both 25 and 50 μM (Fig. 1C). Unlike TNF-α, both LPS and ANG II produce prolonged inhibition of protein synthesis over a 24-h period. ANG II has been shown to attenuate protein synthesis in myotubes through an increased phosphorylation of eIF2α (11), and as shown in Fig. 2, A and B, the increased phosphorylation of both PKR and eIF2α induced by ANG II was completely attenuated by HMB, which itself had no effect on phosphorylation of either PKR or eIF2α. ANG II also increased phosphorylation of the elongation factor eEF2 at Thr⁵⁶ (Fig. 2C), which would inhibit its activity by preventing interaction with the ribosome (5). HMB completely attenuated the phosphorylation of eEF2, which would increase the capacity for translation elongation. The effect on eEF2 is probably due to the ability of HMB to stimulate phosphorylation of mTOR (Fig. 3), which would activate the 70-kDa ribosomal S6 kinase (p⁷⁰⁵⁸⁰), and this in turn would phosphorylate and inactivate eEF2 kinase (28). ANG II (Fig. 3A), TNF-α (Fig. 3B), and LPS (Fig. 3C) had no significant effect on phosphorylation of mTOR, and there was no further change in the presence of HMB. Phosphorylation of mTOR by HMB would also lead to increased phosphorylation of the translation repressor 4E-BP1 (Fig. 4A), leading to a decreased amount of 4E-BP1 associated with eIF4E (Fig. 4B), and an increase in the active eIF4G-eIF4E complex (Fig. 4C). These effects would explain the stimulation of protein synthesis by HMB (Fig. 1). Indeed, the effect of HMB on protein synthesis, either alone or in the presence of LPS, TNF-α, or ANG II, was completely attenuated by cotreatment with rapamycin (10 ng/ml), confirming that activation of mTOR by HMB was the primary stimulus to the increase in protein synthesis (Fig. 3D). Phosphorylation of 4E-BP1 (Fig. 4A), the level of 4E-BP1 associated with eIF4E (Fig. 4B), and the eIF4G-eIF4E complex (Fig. 4C) were not altered by ANG II alone, suggesting that the primary effect of HMB in attenuating the depression of protein synthesis induced by ANG II arose from the ability to attenuate phosphorylation of eIF2α (Fig. 2B) and stimulate phosphorylation of mTOR.

Fig. 4. Effect of ANG II (0.5 μM) on phosphorylation of initiation factor eIF4E-binding protein (4E-BP1) (A), amount of 4E-BP1 associated with eIF4E (B), and amount of eIF4G associated with eIF4E (C) after treatment in the absence or presence of HMB (50 μM). Histograms represent the averages of 3 separate Western blots. *P < 0.001 compared with control. **P < 0.05; *P < 0.01 in the presence of HMB.

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A similar effect was seen with HMB in myotubes incubated with the combination of TNF-α and IFN-γ (Fig. 5). As with ANG II, TNF-α/IFN-γ induced autophosphorylation of PKR (Fig. 5A), and this effect was completely attenuated by HMB. TNF-α/IFN-γ also increased phosphorylation of both eIF2α (Fig. 5B) and eEF2 (Fig. 5C), which also was attenuated by HMB. TNF-α/IFN-γ, although having no effect on the level of phosphorylated 4E-BP1 (Fig. 6A), increased the total 4E-BP1 and the amount of eIF4E bound to 4E-BP1 (Fig. 6B), but HMB had no effect on this. Instead, HMB increased the amount of eIF4G and the eIF4E·eIF4G complex in myotubes exposed to TNF-α and IFN-γ (Fig. 6C). LPS also increased phosphorylation of PKR (Fig. 7A), eIF2α (Fig. 7B), and eEF2 (Fig. 7C) at concentrations between 1 and 100 ng/ml, and this effect also was attenuated by HMB. LPS also had no effect on the level of phosphorylated 4E-BP1 (Fig. 8A), the amount of eIF4E bound to 4E-BP1 (Fig. 8B), or the concentration of the eIF4G·eIF4E complex (Fig. 8C). These results suggest that HMB attenuates the depression of protein synthesis induced by ANG II, TNF-α/IFN-γ, and LPS by attenuating the phosphorylation of eIF2α and eEF2.

The importance of PKR autophosphorylation in the depression of protein synthesis by TNF-α and LPS was determined in myotubes transfected with plasmids that express wild-type or catalytically inactive dominant negative PKR (PKRΔ6) proteins. The results in Fig. 9 show that although TNF-α and LPS depressed protein synthesis in myotubes transfected with either empty plasmid (pcDNA3.1) or wild-type PKR, there was no depression of protein synthesis in myotubes transfected with PKRΔ6. These results confirm the importance of PKR in the depression of protein synthesis by TNF-α and LPS.

DISCUSSION

The results of this study show that HMB can effectively attenuate the depression of protein synthesis in murine myotubes in the presence of ANG II, TNF-α/IFN-γ, or LPS, suggesting a common mechanism of action. Previous studies (9) also have shown HMB to attenuate the depression of protein synthesis in response to PIF, which may contribute to its ability to preserve lean body mass in patients with cancer cachexia (19). The ability of HMB to attenuate the depression of protein synthesis by a disparate group of agents suggests that they may all work through a common mechanism and that HMB may be effective in blocking muscle atrophy in a range of wasting conditions.

Both sepsis and LPS have been shown to decrease muscle protein content, partly by decreasing protein synthesis and translational efficiency (7). The effect of LPS on protein synthesis in gastrocnemius muscle of rats arises from a decrease in translational efficiency demonstrated by a transient increase in binding of 4E-BP1 with eIF4E, a transient decrease in the phosphorylated γ-form of 4E-BP1, and a sustained decrease in the amount of eIF4G associated with eIF4E (15). In contrast to the current in vitro study, the amount of eIF2α in

Fig. 5. Effect of TNF-α (50 ng/ml) + IFN-γ (10 ng/ml) on phosphorylation of PKR (A), eIF2α (B), and eEF2 (C) in murine myotubes 2 h after treatment in the absence or presence of HMB (50 μM). The total forms acted as loading controls. Representative Western blots are shown, and histograms represent the averages of 3 separate experiments. *P < 0.001 compared with control. †P < 0.05; ‡P < 0.01; §P < 0.001 in the presence of HMB.
the phosphorylated form in skeletal muscle did not change, though there was a reduction in the activity of eIF2β, and there was a twofold increase in amount of phosphorylated eIF2α in the liver. The effect of sepsis could be attributed to formation of TNF-α, since the effect on protein synthesis could be reversed by TNF-α binding protein (7). Acute treatment of rats with TNF-α has been shown to depress protein synthesis and increase protein degradation in red-type muscles such as soleus, whereas few effects are seen in white-type muscles (12). However, paradoxically, in tumor-bearing rats the reduced protein synthetic rate in skeletal muscle was increased toward normal after administration of TNF-α (26). A number of in vitro studies using either myotubes or skeletal muscle explants have failed to show that TNF-α induced protein loss, even when used at high concentrations (21). However, in murine myotubes when TNF-α was combined with IFN-γ, there was a large reduction in the level of the myofibrillar protein myosin (1). TNF-α alone and in combination with IFN-γ had a major effect on depression of protein synthesis in murine myotubes after 2 h of incubation. The effect of TNF-α on signaling pathways in vitro is different from that found in vivo (16). Thus, although TNF-α has been shown to decrease the activity of eIF2B in muscle of rats, there was no change in the phosphorylation state of eIF2α compared with this in vitro study, where TNF-α induced autophosphorylation of PKR and increased phosphorylation of eIF2α. In addition, in vivo there was decreased phosphorylation of 4E-BP1, resulting in an increased binding of eIF4E, and a decreased amount of eIF4E associated with eIF4G (16), whereas in vitro there was no effect on phosphorylation of 4E-BP1, but there was an increased binding of eIF4E to 4E-BP1 through an increase in total 4E-BP1.

ANG II also has been shown to depress protein synthesis by activation of PKR, leading to an increased phosphorylation of eIF2α (11), an effect also seen with LPS. LPS has been shown to rapidly induce activation of PKR in rat primary microglia (8), but this is the first report of activation in muscle cells. As with PIF and ANG II (11), activation of PKR is essential for the depression of protein synthesis by TNF-α and LPS, since it was completely attenuated in myotubes transfected with mutant PKRΔ6, which is catalytically inactive and unable to phosphorylate eIF2α (11). This shows that phosphorylation of eIF2α is the most important step in the depression of protein synthesis by TNF-α and LPS. The mechanism by which ANG II, LPS, and TNF-α/IFN-γ all lead to activation of PKR is not known, but separate studies suggest that they all activate a common upstream signaling mechanism. This signaling pathway must also be activated by PIF, since HMB also has been shown to attenuate autophosphorylation of PKR and the subsequent phosphorylation of eIF2α in response to PIF (9).

The other common step by which ANG II, LPS, and TNF-α/IFN-γ reduce the efficiency of translation initiation and thus depress global protein synthesis is through an increased phosphorylation of eEF2, which will result in a reduction in protein synthesis.
Treatment with HMB attenuates the increased phosphorylation of eEF2, possibly by the ability to activate mTOR. eEF2 kinase is phosphorylated and inactivated by p70S6k, which in turn is activated by mTOR (28). Activation of mTOR by HMB leads to increased phosphorylation of 4E-BP1, resulting in less binding of eIF4E and an increase in the eIF4E/eIF4G complex, which promotes protein synthesis (7). This change is likely to be responsible for the stimulation of protein synthesis by HMB, which we have previously suggested to occur through the mTOR/p70S6k pathway (9). At least part of the ability of elongation by decreasing its affinity for the ribosome (5). Treatment with HMB attenuates the increased phosphorylation of eEF2, possibly by the ability to activate mTOR. eEF2 kinase is phosphorylated and inactivated by p70S6k, which in turn is activated by mTOR (28). Activation of mTOR by HMB leads to increased phosphorylation of 4E-BP1, resulting in less binding of eIF4E and an increase in the eIF4E·eIF4G complex, which promotes protein synthesis (7). This change is likely to be responsible for the stimulation of protein synthesis by HMB, which we have previously suggested to occur through the mTOR/p70S6k pathway (9). At least part of the ability of
HMB to attenuate the depression of protein synthesis induced by ANG II, TNF-α, and LPS arises through activation of the mTOR pathway, since the effect was attenuated by rapamycin, an inhibitor of mTOR phosphorylation. A similar effect has been observed in myotubes exposed to PIF and in the soleus muscle of cachexic mice bearing the MAC16 tumor (9).

However, ANG II, LPS or TNF-α/H9251s also results from the attenuation of PKR autophosphorylation of the eIF4E complex, suggesting that the ability of HMB to attenuate the depression of protein synthesis by these agents also results from the attenuation of PKR autophosphorylation and the subsequent phosphorylation of eIF2α.

The ability of HMB to attenuate the depression of protein synthesis in skeletal muscle induced by ANG II, LPS, and TNF-α suggests that it may be effective in countering the loss of muscle mass in CHF, sepsis, and HIV/AIDS. Indeed, clinical studies in patients with HIV support the effectiveness of HMB in preserving lean body mass (6), and more trials are warranted.

**GRANTS**

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**REFERENCES**


