Mechanism of attenuation of muscle protein degradation induced by tumor necrosis factor-α and angiotensin II by β-hydroxy-β-methylbutyrate

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Eley HL, Russell ST, Tisdale MJ. Mechanism of attenuation of muscle protein degradation induced by tumor necrosis factor-α and angiotensin II by β-hydroxy-β-methylbutyrate. Am J Physiol Endocrinol Metab 295: E1417–E1426, 2008. First published October 7, 2008; doi:10.1152/ajpendo.90567.2008.—Both tumor necrosis factor-α (TNF-α)/interferon-γ (IFN-γ) and angiotensin II (ANG II) induced an increase in total protein degradation in murine myotubes, which was completely attenuated by treatment with β-hydroxy-β-methylbutyrate (HMB; 50 μM). There was an increase in formation of reactive oxygen species (ROS) within 30 min, as well as an increase in the activity of both caspase-3 and -8, and both effects were attenuated by HMB. Moreover, inhibitors of caspase-3 and -8 completely attenuated both ROS formation and total protein degradation induced by TNF-α/IFN-γ and ANG II. There was an increased autophosphorylation of double-stranded RNA-dependent protein kinase (PKR), which was attenuated by the specific caspase-3 and -8 inhibitors. Neither ROS formation or protein degradation occurred in myotubes expressing a catalytically inactive PKR variant, PKRΔ6, in response to TNF-α/IFN-γ, compared with myotubes expressing wild-type PKR, although there was still activation of caspase-3 and -8. HMB also attenuated activation of PKR, suggesting that it was important in protein degradation. Formation of ROS was attenuated by rotenone, an inhibitor of the mitochondrial electron transport chain, nitro-arginine methyl ester, an inhibitor of nitric oxide synthase, and SB 203580, a specific inhibitor of p38 mitogen-activated protein kinase (p38 MAPK), which also attenuated total protein degradation. Activation of p38 MAPK by PKR provides the link to ROS formation. These results suggest that TNF-α/IFN-γ and ANG II induce muscle protein degradation by a common signaling pathway, which is attenuated by HMB, and that this involves the initial activation of caspase-3 and -8, followed by autophosphorylation and activation of PKR, which then leads to increased ROS formation via activation of p38 MAPK. Increased ROS formation is known to induce protein degradation through the ubiquitin-proteasome pathway.

LOSS OF SKELETAL MUSCLE MASS IS ASSOCIATED with an increased morbidity and mortality and is found in a number of conditions, including cancer (15), diabetes (21), sepsis (34), renal failure (22), and disuse (33). Muscle mass loss also occurs in the elderly (sarcopenia), where it leads to diminished strength and exercise capacity (11). Muscle mass loss arises from an imbalance between the rates of protein synthesis and degradation. The ubiquitin-proteasome proteolytic pathway has been shown to be responsible for the increased protein degradation seen in cancer (15), diabetes (21), sepsis (34), renal failure (22), and disuse atrophy (33). Induction of the ubiquitin-proteasome pathway by a range of agents, including proteolysis-inducing factor (PIF) (37), cytokines such as tumor necrosis factor-α (TNF-α) (18), angiotensin II (ANG II) (27), and reactive oxygen species (ROS) (19), all involve activation of the transcription factor nuclear factor-κB (NF-κB), suggesting a common mechanism of gene regulation. A further common signaling molecule linking these stimuli is the formation of ROS. We have recently shown (26) that transient formation of ROS is critical in the induction of protein degradation by PIF and ANG II through an NF-κB-mediated induction of the ubiquitin-proteasome pathway. In addition, muscle wasting induced by TNF-α is also associated with an increased oxidative stress (3), as is hindlimb unloading in rats (16). Mice lacking the major antioxidant enzyme Cu,Zn superoxide dismutase also show a dramatic acceleration of age-related loss of skeletal muscle mass (23). The mechanism for formation of ROS is not known.

Activation of the double-stranded RNA-dependent protein kinase (PKR) is also required for muscle atrophy induced by PIF and ANG II (10). Activation of PKR leads to depression of protein synthesis through phosphorylation of eukaryotic initiation factor 2 (eIF2) on the α-subunit, reducing the rate of translation initiation, and also to induction of protein degradation through an increased nuclear binding of NF-κB. Activation of PKR may also lead to an increased ROS production, although this has not been determined.

If a number of diverse agents induce a common mechanism of muscle atrophy, then it should be possible to counteract these effects with a single agent. The leucine metabolite β-hydroxy-β-methylbutyrate (HMB) has been shown to attenuate muscle wasting in cancer (20), acquired immunodeficiency syndrome (AIDS) (4), and aging (35). In cancer-induced muscle atrophy, HMB has been shown to attenuate both the depression of protein synthesis and the increase in protein degradation (30), whereas in vitro studies have shown that it attenuates the depression of protein synthesis induced by both PIF and ANG II (8) and the induction of protein degradation in murine myotubes induced by PIF (31). HMB has been shown to attenuate the PIF-induced autophosphorylation of PKR, as well as increase the active eIF4G·eIF4E complex in murine myotubes (8), both of which would increase protein synthesis. If PKR is involved in ROS formation, then if HMB attenuates activation of PKR, it also is likely to attenuate ROS formation, which would link its activity in restoration of lean body mass in cancer cachexia, sarcopenia, and AIDS if the latter is produced by TNF-α.

The current study investigates the ability of HMB to attenuate the increase in protein degradation in murine myotubes induced by TNF-α as a model of human immunodeficiency.
virus (HIV)/AIDS and by ANG II as a model of cachexia in congestive heart failure (CHF) (1).

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 from ARC (Cardiff, UK). Hybond A nitrocellulose membranes and enhanced chemiluminescence (ECL) development kits were obtained from Amersham Biosciences (Little Chalfont, UK). Mouse monoclonal antibody to phospho-p38 mitogen-activated protein kinase (MAPK) (Thr180/Tyr182) and rabbit polyclonal antiserum to total p38 MAPK were purchased from New England Biolabs (Hitchin, UK). Phosphosafe extraction reagent and the p38 MAPK inhibitor SB 203580 were obtained from Merck Eurolab (Lutterworth, UK), and peroxidase-conjugated goat anti-rabbit antibody was purchased from Dako (Cambridge, UK). ANG II, TNF-α, and IFN-γ were purchased from Sigma-Aldrich (Dorset, UK). The caspase-3 and -8 substrates and inhibitors were purchased from Biomol International (Devon, 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. They were differentiated into myotubes by replacing the growth medium with DMEM containing 2% HS, with the medium being changed every 2 days when they reached about 80% confluency. Differentiation occurred within 3–5 days. Myoblasts were transfected with the plasmid vector pcDNA3 containing both wild-type PKR (a gift from Dr. G. N. Barber, University of Miami School of Medicine, Miami, FL), as described previously (10). Myotubes were formed by allowing confluent cultures of myoblasts to fuse as described above.

Measurement of protein degradation in myotubes. The method for the determination of protein degradation in murine myotubes has been described previously (15). All experiments were carried out in six-well multiwell dishes. Myotubes were labeled 24 h before experimentation with L-[2,6-3H]phenylalanine (26.6 MBq/mmol), washed extensively with PBS, and chased in DMEM lacking phenol red for 2 h to allow degradation of short-lived proteins. HMB or other inhibitors were then added, and after 2 h they were then incubated with LPS, TNF-α, or TNF-α plus IFN-γ or ANG II at the concentrations indicated, together with 2 mM nonlabeled phenylalanine, for 24 h. Protein degradation was determined from the radioactivity released into the medium in relation to the total radioactivity incorporated into the myotubes.

Determination of caspase activity. The activity of caspase-3 and -8 in murine myotubes was determined by the release of 7-amino-4-methylcoumarin (AMC) and 7-amino-4-trifluormethylcoumarin (AFC) from the specific substrates AcDEVD-AMC and Z-IEFD-AFC, respectively. Myotubes were homogenized in lysis buffer A (150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 2 mM EGTA, 0.25% sodium orthovanadate, 20 mM NaF, and 1% protease inhibitor mixture) and were left at 4°C for 10 min, followed by centrifugation at 15,000 g for 10 min, followed by centrifugation at 15,000 g for 15 min. The supernatant (50 μg of protein) was incubated with either the caspase-3 or -8 substrate (10 Fig. 1. Effect of tumor necrosis factor-α (TNF-α; 50 ng/ml), alone or in the presence of interferon-γ (IFN-γ; 10 ng/ml), and angiotensin II (ANG II) on total protein degradation in murine myotubes after 24 h of incubation (A and B) and reactive oxygen species (ROS) production after 30 min of incubation (C and D) in the absence (●) or presence (○) of β-hydroxy-β-methylbutyrate (HMB; 50 μM) added 1 h before the stimulus. The results are averages of 3 separate replicate experiments. *P < 0.05; †P < 0.01; ‡P < 0.001 compared with control. *P < 0.01; †P < 0.001 in the presence of HMB.

AJP-Endocrinol Metab • VOL 295 • DECEMBER 2008 • www.ajpendo.org
µM) for 1 h in the absence or presence of the caspase-3 (AcDEVD-CHO) or caspase-8 (IEFTD-CHO) inhibitors (100 µM). The increase in fluorescence due to AMC was determined at an excitation wavelength of 370 nm and an emission wavelength of 430 nm, whereas the increase in fluorescence due to AFC was measured with an excitation wavelength of 400 nm and an emission of 505 nm. The difference in values in the absence and presence of the caspase inhibitors was a measure of activity.

**Determination of ROS levels in myotubes.** This method has been described previously (26) and depends on the oxidation of dichlorodihydrofluorescein to the highly fluorescent dichlorofluorescein. Myotubes were treated with the various agonists in DMEM without phenol red, washed with PBS, and incubated in fresh medium containing 0.1 µM 2,7-dichlorodihydrofluorescein diacetate for 30 min. They were then washed in PBS, sonicated at 4°C, and centrifuged at 2,800 g for 10 min. The fluorescence of an aliquot of the supernatant was determined at an excitation wavelength of 480 nm and an emission wavelength of 510 nm.

**Western blot analysis.** Myotubes were treated 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 12% SDS-PAGE at 180 V for 1 h, followed by transference to 0.45-m nitrocellulose membranes, which were then blocked with 5% Marvel in Tris-buffered saline, pH 7.5, at 4°C overnight. The primary antibody was used at a dilution of 1:1,000, and the secondary antibody activity (7). The results in Fig. 2 show that TNF-α (Fig. 2A) and ANG II (Fig. 2B) alone had no effect on ROS production. These results suggest that HMB attenuates a common signaling pathway induced by each of these agents leading to ROS formation.

One of the upstream signaling pathways thought to be involved in protein degradation is the induction of caspase activity (7). The results in Fig. 2 show that TNF-α (A and B)
and ANG II (C and D) both induced an increase in both caspase-3 (A and C) and caspase-8 activity (B and D) within 2 h of treatment and that this was maintained over the 4-h period of investigation. The effect was specific, since it was attenuated by both caspase inhibitors and also was completely attenuated by HMB. To determine which caspase is upstream of the other, we determined the effect of the caspase-3 inhibitor on caspase-8 activity (Fig. 2E), and the effect of the caspase-8 inhibitor on caspase-3 activity (Fig. 2F) was determined after treatment of myotubes with ANG II or TNF-α. Whereas the caspase-3 inhibitor had no effect on the caspase-8 response (Fig. 2E), the caspase-8 inhibitor completely attenuated the caspase-3 response (Fig. 2F). These results suggest that caspase-8 is upstream of caspase-3.

Both caspase-3 and -8 inhibitors completely attenuated total protein degradation induced by ANG II (Fig. 3A) and TNF-α/IFN-γ (Fig. 3B), showing the importance of caspase activation to protein degradation. Furthermore, ROS production induced by TNF-α and TNF-α/IFN-γ (Fig. 3C) and ANG II (Fig. 3D) was completely attenuated by the caspase-3 and -8 inhibitors, suggesting that caspase-3 and -8 are upstream signals leading to increased ROS formation.

To determine whether activation of PKR also was involved in this process, we investigated the effect of the caspase-3 and -8 inhibitors on autophosphorylation of PKR in response to ANG II (Fig. 4, A and B) and TNF-α/IFN-γ (Fig. 4C). Whereas ANG II and TNF-α/IFN-γ induced autophosphorylation of PKR in the absence of the caspase inhibitors, there was complete attenuation in the presence of either the caspase-3 or -8 inhibitor. These results show that caspase activation also leads to activation of PKR.

Previous studies (10) have shown that activation of PKR is essential for the induction of protein degradation by ANG II. The data in Fig. 4D show that this also applies to TNF-α, because although protein degradation occurred in myotubes transfected with empty plasmid (pcDNA3) and wild-type PKR, it was completely attenuated in myotubes transfected with PKRΔ6, a mutant PKR, which lacks six amino acids (361–366) between catalytic domains IV and V, rendering it unable to autophosphorylate or catalyze phosphorylation of protein substrates (10).

To determine whether activation of PKR occurs above or below ROS production, we determined the effect of TNF-α/IFN-γ and ANG II in myotubes transfected with PKRΔ6. In contrast with myotubes transfected with empty plasmid (pcDNA 3.1) and wild-type PKR, there was no increase in ROS formation in response to ANG II or TNF-α/IFN-γ in myotubes transfected with PKRΔ6 (Fig. 5A). In response to the agonists, HMB completely attenuated ROS formation in both

Fig. 3. A and B: effect of ANG II (A) and TNF-α (50 ng/ml) + IFN-γ (10 ng/ml) (B) on total protein degradation in murine myotubes when administered alone (●) or in the presence of the caspase-3 (■) and caspase-8 (●) inhibitors (both at 100 μM), added 1 h before the catabolic stimulus. C and D: effect of TNF-α (50 ng/ml) + IFN-γ (10 ng/ml) (C) and ANG II (0.5 μM) (D) on ROS formation in murine myotubes after 30 min of incubation in the presence of caspase-3 and caspase-8 inhibitors (both at 100 μM). The results are averages of 3 separate experiments and are expressed by taking basal fluorescence of control cells as 100%. *P < 0.001 compared with control. †P < 0.001 in the presence of caspase inhibitors.
myotubes containing pcDNA 3.1 and wild-type PKR down to the values found in PKRΔ6.

Myotubes transfected with PKRΔ6 still, however, showed the increase in activity of caspase-3 (Fig. 5B) and caspase-8 (Fig. 5C) in response to TNF-α. These results confirm that the pathway for protein degradation induced by ANG II and TNF-α is the same and involves the initial activation of caspases-3 and -8, followed by autophosphorylation of PKR, which in turn leads to an increased ROS formation. HMB attenuates this process by inhibiting activation of caspase-3 and -8.

Previous studies (26) had suggested that ROS formation induced by ANG II occurred through activation of NAD(P)H oxidase. However, there was no increase in mRNA expression of the NAD(P)H oxidase subunits p40phox, p91phox, or p47phox in response to ANG II (results not shown). TNF-α-induced ROS generation is mediated by a Rac-cytosolic PLA2 (cPLA2)-5-lipoxygenase (LOX)-linked cascade (36), and formation of ROS by ANG II also requires Rac/PLA2/LOX (27).

The results in Fig. 6 show complete attenuation of ROS formation in response to ANG II and TNF-α/IFN-γ by rotenone (100 μM), an inhibitor of the mitochondrial electron transport chain (A and B), by nitro-l-arginine methyl ester (L-NAME; 10 μM), an inhibitor of nitric oxide synthase (NOS; C and D), and by SB 203580 (10 μM), a selective inhibitor of p38 MAPK (C and D). Activation of p38 MAPK could provide a link between activation of PKR and ROS formation. Western blotting (Fig. 7) showed an increased ratio of phosphorylated to total p38 MAPK after treatment with either TNF-α/IFN-γ (A) or ANG II (B) in myotubes transfected with either pcDNA3.1 or wild-type PKR, but not in those transfected with PKRΔ6. These results confirm that activation of PKR is responsible for the activation of p38 MAPK. This was supported by the data in Fig. 7, which show no change in the ratio of phosphorylated to total p38 MAPK in either transfected (A and B) or nontransfected (C and D) myotubes treated with HMB. The ability of HMB to inhibit activation of p38 MAPK would explain its ability to block ROS formation in response to ANG II and TNF-α/IFN-γ (Fig. 1, C and D).

In addition, treatment with the p38 MAPK inhibitor SB 203580 (10 μM) also completely attenuated total protein degradation induced by ANG II and TNF-α/IFN-γ (Fig. 7E and ANG II (Fig. 7F), showing that ROS formation through p38 MAPK also is essential for induction of protein degradation. Previous studies have shown that protein degradation induced by TNF-α (3) and ANG II (26) was completely attenuated by treatment with antioxidants.

**Discussion**

The results of the current study show that HMB effectively attenuates the increase in protein degradation induced by TNF-α/IFN-γ and ANG II, suggesting that it may be effective in preserving lean body mass in HIV/AIDS (4) and CHF. Previous studies have shown HMB to attenuate the depression of protein synthesis and increase in protein degradation in murine myotubes transfected with pcDNA 3.1, wild-type PKR, and a catalytically inactive PKR variant, PKRΔ6, after 24 h of incubation. The results in Fig. 6 show complete attenuation of ROS formation in response to ANG II and TNF-α/IFN-γ by rotenone (100 μM), an inhibitor of the mitochondrial electron transport chain (A and B), by nitro-l-arginine methyl ester (L-NAME; 10 μM), an inhibitor of nitric oxide synthase (NOS; C and D), and by SB 203580 (10 μM), a selective inhibitor of p38 MAPK (C and D). Activation of p38 MAPK could provide a link between activation of PKR and ROS formation. Western blotting (Fig. 7) showed an increased ratio of phosphorylated to total p38 MAPK after treatment with either TNF-α/IFN-γ (A) or ANG II (B) in myotubes transfected with either pcDNA3.1 or wild-type PKR, but not in those transfected with PKRΔ6. These results confirm that activation of PKR is responsible for the activation of p38 MAPK. This was supported by the data in Fig. 7, which show no change in the ratio of phosphorylated to total p38 MAPK in either transfected (A and B) or nontransfected (C and D) myotubes treated with HMB. The ability of HMB to inhibit activation of p38 MAPK would explain its ability to block ROS formation in response to ANG II and TNF-α/IFN-γ (Fig. 1, C and D).

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![Fig. 4. Effect of caspase-3 and -8 inhibitors (100 μM) on autophosphorylation (p) of double-stranded RNA-dependent protein kinase (PKR) in response to ANG II (A and B) and TNF-α (50 ng/ml) + IFN-γ (10 ng/ml) (C) after 4 h of incubation. The caspase inhibitors were added 1 h before the stimulus. The densitometric analysis represents the average of 3 separate Western blots. The agonist alone is represented by filled bars, whereas values in the presence of the caspase inhibitors are represented by open bars. *P < 0.001 compared with control. **P < 0.001 in the presence of caspase inhibitors.](http://ajpendo.physiology.org/10.220.33.4)
myotubes in response to PIF (8), and clinical studies have shown it to increase lean body mass in patients with cancer cachexia (20). The present results suggest that HMB attenuated a common pathway in protein degradation involving activation of caspases-3 and -8, autophosphorylation of PKR, and formation of ROS. The results of this study provide a potential mechanism by which HMB attenuates muscle wasting in aging (35), which probably involves a similar mechanism involving formation of ROS (23).

ROS formation has been shown previously to play an important role in protein degradation induced by PIF, ANG II (26), and TNF-α (3). ROS can increase protein degradation by activation of caspase-3 (24), NF-kB (18), and the Foxo signaling pathway in muscle cells (12). Hydrogen peroxide has been shown to induce muscle protein degradation through the ubiquitin-proteasome pathway (13) and increases gene expression of the ubiquitin-ligase (E3) atrogin 1/MAFbx (17). For TNF-α-induced ROS generation, a Rac-cPLA2-5LOX-linked cascade is employed (36), and the current study suggests activation of both caspases-3 and -8, as well as PKR, are also important upstream signaling events. TNF-α and ANG II both increased the activity of caspases-3 and -8, and this effect is important in total protein degradation by these agents, since it was completely attenuated by both caspase-3 and -8 inhibitors. Studies with specific caspase inhibitors suggest that the initial stimulus led to activation of caspase-8, and that this in turn led to activation of caspase-3. In addition, ROS formation by each of these agents was also attenuated by the caspase-3 and -8 inhibitors, suggesting that caspase activation was important in ROS formation. Also ANG II and TNF-α induced activation (autophosphorylation) of PKR, and this was attenuated by both the caspase-3 and -8 inhibitors.

Fig. 5. A: effect of ANG II (0.5 μM) and TNF-α (50 ng/ml) + IFN-γ (10 ng/ml) on ROS formation in murine myotubes transfected with pcDNA 3.1 (filled bars), wild-type PKR (open bars), and PKRΔ6 (hatched bars) in the absence or presence of HMB (50 μM). B and C: effect of TNF-α on caspase-3 and caspase-8 activity in murine myotubes transfected with pcDNA 3.1 (filled bars), wild-type PKR (open bars), and PKRΔ6 (hatched bars) in the absence and presence of their specific inhibitors after 2 h of incubation. *P < 0.001 compared with control. **P < 0.001 compared with either wild-type PKR or pcDNA 3.1 or in the presence of the caspase inhibitor or HMB.

Caspase-3 and -8 inhibitors, suggesting that activation of caspase-3 and -8 is important in total protein degradation by these agents, since it was completely attenuated by both caspase-3 and -8 inhibitors. Studies with specific caspase inhibitors suggest that the initial stimulus led to activation of caspase-8, and that this in turn led to activation of caspase-3. In addition, ROS formation by each of these agents was also attenuated by the caspase-3 and -8 inhibitors, suggesting that caspase activation was important in ROS formation. Also ANG II and TNF-α induced activation (autophosphorylation) of PKR, and this was attenuated by both the caspase-3 and -8 inhibitors, suggesting that activation of caspase was involved in the activation of PKR.

Fig. 5. A: effect of ANG II (0.5 μM) and TNF-α (50 ng/ml) + IFN-γ (10 ng/ml) on ROS formation in murine myotubes transfected with pcDNA 3.1 (filled bars), wild-type PKR (open bars), and PKRΔ6 (hatched bars) in the absence or presence of HMB (50 μM). B and C: effect of TNF-α on caspase-3 and caspase-8 activity in murine myotubes transfected with pcDNA 3.1 (filled bars), wild-type PKR (open bars), and PKRΔ6 (hatched bars) in the absence and presence of their specific inhibitors after 2 h of incubation. *P < 0.001 compared with control. **P < 0.001 compared with either wild-type PKR or pcDNA 3.1 or in the presence of the caspase inhibitor or HMB.

Studies with specific caspase inhibitors suggest that the initial stimulus led to activation of caspase-8, and that this in turn led to activation of caspase-3. In addition, ROS formation by each of these agents was also attenuated by the caspase-3 and -8 inhibitors, suggesting that caspase activation was important in ROS formation. Also ANG II and TNF-α induced activation (autophosphorylation) of PKR, and this was attenuated by both the caspase-3 and -8 inhibitors, suggesting that activation of caspase was involved in the activation of PKR.

Activation of PKR leads to ROS production, since neither ANG II or TNF-α induced ROS formation in myotubes transfected with the mutant PKRΔ6, which cannot undergo autophosphorylation, although there was still activation of caspases-3 and -8. As previously shown for PIF and ANG II (10), activation of PKR is essential for protein degradation induced by TNF-α/IFN-γ, since protein degradation was completely attenuated in myotubes transfected with the mutant PKRΔ6. These results suggest that the signaling pathway for protein degradation by a range of agents is the same and involves activation of PKR.

These results suggest that the order of the signaling cascade is as shown in Fig. 8. Thus TNF-α and ANG II initially activate caspase-8 and then caspase-3, leading to activation of PKR and, subsequently, ROS formation. Previous studies (26) have shown that ROS cause activation of NF-kB, leading to increased transcription of key genes of the ubiquitin-proteasome pathway, and degradation of myofibrillar proteins. The mechanism for the activation of PKR has not been investigated, but other studies (32) investigating neural death in response to aggregated β-amyloid peptide have shown caspase-3 to be activated by caspase-8 and that caspase-3 was responsible for activation of PKR. Caspases-3, -7, and -8 have been shown to cleave PKR at Asp51, releasing the kinase domain from the control of the regulatory amino-terminal domain and leading to phosphorylation of eIF2α at Ser51 (28). ROS formation induced by TNF-α is known to involve release of arachidonic acid through activation of cPLA2 (36). p38 MAPK is known to be required for the phosphorylation and activation of cPLA2 (14), and in the current study, the specific p38 MAPK inhibitor (SB 203580) was found to attenuate ROS production by TNF-α and ANG II, suggesting that this pathway is operative. In addition, SB 203580 also attenuated total protein degradation.
tion induced by TNF-α/IFN-γ and ANG II, showing the importance of this step to protein degradation. Activation (phosphorylation) of p38 MAPK was seen in myotubes containing wild-type PKR in response to TNF-α but not in those containing the mutant PKR. The most likely mechanism by which this could occur via PKR would be through activation of MAPK kinase 6 (MKK6), which would then activate p38 MAPK (29).

This study provides a mechanism by which HMB attenuates the increase in protein degradation in myotubes in response to ANG II and TNF-α by attenuation of activation of caspases-3 and -8 and the subsequent attenuation of the activation of PKR and ROS formation. The mechanism by which HMB attenuates caspase-3 and -8 activation is not known. The involvement of the apoptotic effector caspase-3 and the initiator caspase-8 suggests that the mechanism of induction of muscle protein degradation is closely associated with apoptosis.

The ability of HMB to attenuate activation of PKR in response to ANG II, LPS, and TNF-α/IFN-γ would provide a mechanism for the inhibition of protein degradation by inhibiting activation of NF-κB and the subsequent increase in proteasome expression and activity (10). HMB may attenuate activation of PKR through inhibition of caspase activation or through induction of the protein phosphatase (PP1), which dephosphorylates PKR in analogy with the effect of leucine (9). Attenuation of PKR autophosphorylation would also counteract the impairment of translation initiation by preventing phosphorylation of eIF2 on the α-subunit.

Activation of p38 MAPK by TNF-α has been shown to stimulate expression of the ubiquitin ligase atrogin1/MAFbx in skeletal muscle, and it is therefore an essential step in the degradative pathway (17).

Reactive nitrogen species (RNS) also have been shown to induce activation of NF-κB, leading to protein degradation in skeletal muscle cells (2). In addition to ROS, induction of NOS by TNF-α has been shown to be important in both wasting and the molecular abnormalities in skeletal muscle (3). ANG II also stimulates production of RNS, such as NO and peroxynitrite, by activation of inducible NOS (5). NO production by TNF-α has been reported to stimulate loss of MyoD mRNA in skeletal muscle through formation of peroxynitrite by conjugation with superoxide (6). In the current study, the NOS inhibitor L-NAME attenuated ROS production induced not only by TNF-α but also by ANG II, suggesting that NO production contributes to the measured ROS response.

The results of this study show that the intracellular signaling pathway involved in muscle protein degradation induced by TNF-α and ANG II is the same, suggesting that agents used in the treatment of one type of muscle atrophy also should be effective in other types, providing that they inhibit somewhere in the cascade below the receptor. This would explain the ability of HMB to counter muscle atrophy in a range of conditions including cancer (20), HIV/AIDS (4), and aging (35). The ability of HMB to attenuate not only the rise in protein degradation in skeletal muscle but also the depression of protein synthesis makes it an ideal agent for the treatment of muscle-wasting diseases.

**GRANTS**

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Fig. 7. A–D: Western blots showing the ratio of phosphorylated to total p38 mitogen-activated protein kinase (MAPK) in myotubes transfected with either pcDNA3.1 (filled bars), wild-type PKR (open bars), or PKRΔ6 (hatched bars) after treatment with TNF-α (50 ng/ml) + IFN-γ (10 ng/ml) (A) or ANG II (0.5 μM) (B) for 2 h in the absence or presence of HMB (50 μM). The effect of HMB (50 μM) on the ratio of phosphorylated to total p38 MAPK after treatment with ANG II (C) and TNF-α/IFN-γ (D) on nontransfected myotubes is shown. The densitometric analysis represents the average of 3 separate Western blots. *P < 0.001 compared with control. †P < 0.001 in the presence of HMB.

E: effect of TNF-α (50 ng/ml) + IFN-γ (10 ng/ml) (E) and ANG II (0.5 μM) (F) on total protein degradation in murine myotubes after 24 h in either the absence (●) or presence (■) of the p38 MAPK inhibitor SB 203580 (10 μM). *P < 0.001 compared with control; †P < 0.01; ‡P < 0.001 in the presence of SB 203580.
Fig. 8. Signaling cascade initiated by TNF-α and ANG II leading to depression of protein synthesis and increase in protein degradation in skeletal muscle and the effect of HMB on this process. Increases (↑) or decreases (↓) in activity are indicated.

REFERENCES


