Effect of branched-chain amino acids on muscle atrophy in cancer cachexia

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In the present study, the BCAAs (branched-chain amino acids) leucine and valine caused a significant suppression in the loss of body weight in mice bearing a cachexia-inducing tumour (MAC16), producing a significant increase in skeletal muscle wet weight, through an increase in protein synthesis and a decrease in degradation. Leucine attenuated the increased phosphorylation of PKR (double-stranded-RNA-dependent protein kinase) and eIF2α (eukaryotic initiation factor 2α) in skeletal muscle of mice bearing the MAC16 tumour, due to an increased expression of PP1 (protein phosphatase 1). Weight loss in mice bearing the MAC16 tumour was associated with an increased amount of eIF4E bound to its binding protein 4E-BP1 (eIF4E-binding protein 1), and a progressive decrease in the active eIF4G–eIF4E complex due to hypophosphorylation of 4E-BP1. This may be due to a reduction in the phosphorylation of mTOR (mammalian target of rapamycin), which may also be responsible for the decreased phosphorylation of p70S6K (70 kDa ribosomal S6 kinase). There was also a 5-fold increase in the phosphorylation of eEF2 (eukaryotic elongation factor 2), which would also decrease protein synthesis through a decrease in translation elongation. Treatment with leucine increased phosphorylation of mTOR and p70S6K, caused hyperphosphorylation of 4E-BP1, reduced the amount of 4E-BP1 associated with eIF4E and caused an increase in the eIF4G–eIF4E complex, together with a reduction in phosphorylation of eEF2. These changes would be expected to increase protein synthesis, whereas a reduction in the activation of PKR would be expected to attenuate the increased protein degradation.

Key words: branched-chain amino acid (BCAA), cachexia, double-stranded-RNA-dependent protein kinase (PKR), eukaryotic elongation factor 2 (eEF2), eukaryotic-initiation factor 4E (eIF4E), eukaryotic-initiation factor 4E-binding protein 1 (4E-BP1), leucine.

INTRODUCTION

Muscle protein loss in cancer cachexia is considered to arise from a combination of hypoanabolism together with an increase in catabolism. The primary mechanism for the increase in catabolism has been attributed to an increase in expression and activity of the ubiquitin–proteasome proteolytic pathway [1], but until recently the mechanism underlining the failure to maintain protein synthesis was largely unknown. However, a single mechanism has been proposed to explain the depression in protein synthesis and the increased degradation of myofibrillar proteins in cachexia through the activation, by autophosphorylation, of PKR (double-stranded-RNA-dependent protein kinase) [2]. Activation of PKR by agents such as PIF (proteolysis-inducing factor) and Ang II (angiotensin II) induces phosphorylation of eIF2α (eukaryotic initiation factor 2α), leading to inhibition of translation initiation, through competition with the guanine-nucleotide-exchange factor, eIF2B, preventing the conversion of eIF2 from its GDP-bound state into the active GTP-bound form [3]. Activation of PKR also induces protein degradation in muscle through the induction of the expression and activity of the ubiquitin–proteasome pathway in an NF-κB (nuclear factor κB)-mediated process [2]. Agents such as IGF-1 (insulin-like growth factor-1), which attenuate protein degradation in muscle, also attenuate the activation of PKR [4], suggesting an avenue for therapeutic development in the treatment of muscle-wasting diseases.

Protein synthesis also requires a correctly balanced mixture of amino acids, and a number of studies have noted widespread decreases in the plasma levels of free amino acids in patients with cachexia [5]. The maximum decreases are often found for the BCAAs (branched-chain amino acids) leucine, isoleucine and valine [6]. BCAAs, as well as being integral components of skeletal muscle proteins, are uniquely able to initiate signal transduction pathways that modulate translation initiation [7]. Of the BCAAs, leucine is most potent in stimulating muscle protein synthesis, whereas isoleucine and valine are much less effective [8]. The mechanism for stimulation is through activation of the mRNA-binding steps in translation initiation through hyperphosphorylation of 4E-BP1 (eIF4E-binding protein 1), resulting in the release of eIF4E from the inactive 4E-BP1–eIF4E complex [9]. The freed eIF4E then associates with eIF4G to form the active eIF4F complex. Leucine also stimulates the phosphorylation and thus the activation of p70S6K (70 kDa ribosomal S6 kinase), which also stimulates protein synthesis [8]. The effect of BCAAs on activation of PKR has not been investigated previously, although administration of leucine to rats, which had been fasted for 18 h, did not cause significant alterations in either the activity of eIF2B or the phosphorylation of eIF2α [9].

The aim of the present study was to investigate the effect of BCAAs on muscle wasting and to examine the mechanism, both in vitro, using catabolic stimuli such as PIF, and in vivo, in mice bearing a cachexia-inducing tumour.

EXPERIMENTAL

Materials

FCS (fetal calf serum), HS (horse serum) and DMEM (Dulbecco’s modified Eagle’s medium) were purchased from Life Technologies. L-[2,6-3H]Phenylalanine (specific radioactivity, 1 To whom correspondence should be addressed (email m.j.tisdale@aston.ac.uk).
2.0 TBq/(mmol). Hybond A nitrocellulose membranes, m<sup>7</sup>GTP (7-methyl-GTP)-Sepharose 4B and ECL<sup>®</sup> (enhanced chemiluminescence) development kits were from Amersham Biosciences. Rabbit polyclonal antisera to phospho-eIF2α (Ser<sup>157</sup>) was from Abcam, and rabbit polyclonal antisera to total eIF2α was from Santa Cruz Biotechnology. Rabbit polyclonal antisera to 4E-BP1–eIF4E, phospho-eIF4E (Ser<sup>209</sup>), total eIF4G, phospho-eIF4G (Ser<sup>1108</sup>), phospho-eEF2 (eukaryotic elongation factor 2) (Thr<sup>56</sup>), phospho-mTOR (Ser<sup>2448</sup>), phospho-p70S6k (Thr<sup>389</sup>) and total p70S6k and rabbit monoclonal antibodies to phospho-4E-BP1 (Thr<sup>37/46</sup>) and total PKR were from New England Biolabs. A mouse monoclonal antibody to PPI (protein phosphatase 1) was purchased from Autogen Bioclear. Peroxidase-conjugated goat anti-rabbit and peroxidase-conjugated rabbit anti-mouse antibodies were purchased from Dako. PIF or Ang II was added at the concentrations indicated in the Figures, followed by 2 µl (370 kBq) of L-[2,6-<sup>3</sup>H]phenylalanine from cells prelabelled for 24 h as described previously [13]. After labelling, cells were washed extensively and pre-incubated in fresh medium for 2 h to allow degradation of short-lived proteins. Protein degradation was determined over a 24 h period in medium containing 2 mmol/l unlabelled phenylalanine to prevent re-incorporation of the radiolabel. The radioactivity released into the medium was calculated as a fraction of the total radioactivity incorporated into the myotubes.

**Protein synthesis and degradation in muscle**

The method for the determination of protein synthesis in muscle has been described previously [14]. Protein synthesis was measured by the incorporation of L-[2,6-<sup>3</sup>H]phenylalanine into acid-insoluble material during a 2 h period in which soleus muscles were incubated at 37°C in RPMI 1640 without Phenol Red and saturated with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. After incubation, muscles were rinsed in non-radioactive medium, blotted and homogenized in 4 ml of 2% (v/v) perchloric acid. The rate of protein synthesis was calculated by dividing the amount of protein-bound radioactivity by the amount of acid-soluble radioactivity.

For protein degradation, gastrocnemius muscle was incubated in 3 ml of oxygenated (95% O<sub>2</sub> and 5% CO<sub>2</sub>) Krebs–Hensel buffer (pH 7.4), containing 5 mmol/l glucose and 0.5 mmol/l cycloheximide. The rate of protein degradation was determined by the release of tyrosine [15] over a 2 h period.

**Western blot analysis**

Samples (approx. 10 mg) of gastrocnemius muscle were homogenized in 500 µl of PhosphoSafe<sup>™</sup> extraction reagent and centrifuged at 15 000 g for 15 min. Samples of cytosolic protein (10 µg) were resolved by SDS/PAGE (10% gels, 6% gels for eIF2α or 15% gels for 4E-BP1), and transferred on to 0.45 µm nitrocellulose membranes, which had been blocked with 5% (w/v) non-fat dried milk (Marvel) in PBS (pH 7.5) at 4°C for 1–2 h, and then washed for 15 min in 0.1% Tween 20-buffered saline or PBS/Tween 20, prior to adding the primary antibodies. The primary antibodies were used at a dilution of 1:1000, except for phospho-eIF2α (1:500 dilution) and actin (1:200 dilution) antibodies. The secondary antibodies were used at a dilution of 1:1000. Incubation was for 1 h at room temperature (for actin) or overnight, and development was by ECL<sup>®</sup>. Blots were scanned by a densitometer to quantify differences.

m<sup>7</sup>GTP-Sepharose chromatography

The extent of phosphorylation of 4E-BP1 and the association of 4E-BP1 and eIF4G with eIF4E was determined by Western blotting when eIF4E was extracted from the muscle samples by m<sup>7</sup>GTP-Sepharose 4B-affinity binding. Muscle samples were...
The amino acids (2 mmol/l) were added 2 h prior to PIF (4.2 mmol/l). The concentration of the amino acids in DMEM was: 0.8 mmol/l for Leu, Ile and Val; 0.4 mmol/l for Phe; 0.34 mmol/l for Arg; and 0.2 mmol/l for Met. The results are means ± S.E.M. of three separate determinations.

*P < 0.05, **P < 0.01 or ***P < 0.001 compared with control; †††P < 0.001 compared with PIF alone.

by the phosphatase [16] (Figure 3A). There was no significant effect of salubrinal on protein synthesis inhibition by PIF alone (Figure 3A). Stimulation of protein synthesis by leucine in the absence of PIF was also attenuated by salubrinal, suggesting that this was due, at least in part, to inhibition of eIF2α phosphorylation. This effect appears to be due to attenuation of the activity of PKR, since the ability of leucine to reverse the inhibition of protein synthesis by PIF was enhanced in the presence of a PKR inhibitor [17] up to the value of leucine alone (Figure 3A). Similar results were obtained when Ang II was used to depress protein synthesis (Figure 3B). These results suggest that leucine acts to attenuate activation of PKR by PIF and Ang II. Leucine, but not valine or isoleucine, also partially attenuated the increase in protein degradation induced by PIF (Figure 4). Such an effect would be predicted from its ability to attenuate activation of PKR [2].

To investigate the effect of BCAAs on muscle loss in cachexia, the individual BCAAs were administered daily to mice bearing the MAC16 tumour, and the effects on weight loss and tumour growth were evaluated (Figure 5). Of the three BCAAs, only leucine and valine caused a significant suppression of body weight loss (Figure 5A), whereas only leucine caused an increase in soleus muscle wet weight (Figure 5C). Leucine and valine also produced a significant increase in protein synthesis (Figure 5D), whereas only leucine produced a decrease in protein degradation in skeletal muscle (Figure 5E). Interestingly, leucine and valine produced a small, but significant, inhibition of tumour growth (Figure 5B), an effect also seen with a low-molecular-mass PKR inhibitor [18].

We have shown previously [2] that weight loss induced by the MAC16 tumour is associated with an increased phosphorylation of PKR and eIF2α in gastrocnemius muscle, which contribute to the depression of protein synthesis and protein degradation.
The results shown in Figures 6(A) and 6(B) indicate that leucine attenuated the increased phosphorylation of both PKR and eIF2α, almost to values found in non-tumour-bearing animals, whereas levels of total PKR and eIF2α remained unchanged. The decreased phosphorylation of PKR is accompanied by a 2.5-fold increase in expression of PP1 (Figure 6C) in muscles of mice bearing the MAC16 tumour which were treated with leucine. PKR is a known substrate of PP1 [19].

The effect of weight loss on the amount of eIF4E available for active eIF4G–eIF4E complex formation in gastrocnemius muscle is shown in Figure 7. There was a progressive increase in the amount of 4E-BP1 associated with eIF4E with increasing weight loss (Figure 7A), and a progressive decrease in the formation of the eIF4G–eIF4E complex (Figure 7B), which had an 80% decrease in animals with a weight loss of 20%, compared with those with no weight loss. The decrease in the availability of eIF4E for binding eIF4G resulted from hypophosphorylation of 4E-BP1. There was a 5-fold reduction in 4E-BP1 phosphorylation in animals with a 20% weight loss compared with those without weight loss (Figure 7C). There was no effect of weight loss on phosphorylation of eIF4E (Figure 7D), but a 5-fold increase in phosphorylation of eEF2, with no change in the total amount (Figure 7E), indicating that global protein synthesis was also reduced through a decrease in translation elongation.

Treatment of mice bearing the MAC16 tumour with leucine (Figure 5) reversed these changes in gastrocnemius muscle towards that seen in animals without weight loss (Figure 8). Thus leucine treatment caused a reduction in the amount of 4E-BP1 associated with eIF4E (Figure 8A), and an increase in the eIF4G–eIF4E complex above the value found in non-tumour-bearing animals (Figure 8B). This was due to the hyperphosphorylation of 4E-BP1 (Figure 8C), without an effect on the phosphorylation of eIF4E (Figure 8D). Leucine treatment also reduced the phosphorylation of eEF2 (Figure 8E). These changes would explain the ability of leucine to stimulate protein synthesis in the gastrocnemius muscle.

The effect of weight loss on the expression of the phosphorylated forms of mTOR and p70S6k in the gastrocnemius muscle is shown in Figure 7. As with 4E-BP1 (Figure 7C), there was a progressive reduction in phosphorylation of both mTOR and p70S6k with increasing weight loss, with values at 20% weight loss being only 30% for mTOR (Figure 7F) and 40% for p70S6k (Figure 7G) of the values found in animals without weight loss, without a change on total mTOR or p70S6k. Leucine treatment of mice bearing the MAC16 tumour increased levels of phosphorylated mTOR (Figure 6D) and phosphorylated p70S6k (Figure 6E) in the gastrocnemius muscle up to values found in non-tumour-bearing animals.

**DISCUSSION**

BCAAs comprise 14–18% of the total amino acids in muscle proteins [20], and function not only as building blocks, but also as modulators of protein synthesis. Although BCAAs are known to stimulate protein synthesis and inhibit protein degradation in the skeletal muscle of cachectic rats [21], there have been no studies on the mechanisms involved in this effect. In starvation, the stimulatory effect of leucine on protein synthesis has been attributed to hyperphosphorylation of eIF2α and p70S6k [9], with little effect on the phosphorylation of eIF2α or on the activity of eIF2B. In cachexia, the mechanism appears to be similar to that seen in starvation, with hyperphosphorylation of 4E-BP1 and p70S6k, but in addition leucine has been shown to inhibit phosphorylation of eIF2α through a direct effect on the activation of PKR by phosphorylation.

A previous study [2] has shown an increase in phosphorylation of PKR and eIF2α in gastrocnemius muscle of weight-losing mice bearing the MAC16 tumour. Such a change is concordant...
Effect of BCAAs on cancer cachexia

Figure 5  Effect of administration of BCAAs on muscle loss in cachexia in mice bearing the MA16 tumour

Effect of daily per os administration of leucine (•), isoleucine (▲) and valine (△) (all at 1g/kg of body weight) in comparison with PBS controls (○) on body weight change (A) and tumour volume (B) of mice bearing the MAC16 tumour. At the end of the experiment, mean weight of the soleus muscles (C), the rate of protein synthesis in gastrocnemius muscles (D) and the rate of protein degradation in soleus muscles (E) were determined. The details of the experiment are given in the Experimental section. Values are means ± S.E.M. of six animals per treatment. *P < 0.05, **P < 0.01 and ***P < 0.001 compared with control.

with the ability of hyperphosphorylated PKR to inhibit protein synthesis through an effect on translation initiation and an increase in protein degradation through up-regulation of the ubiquitin–proteasome proteolytic pathway [2]. Muscle atrophy in mice bearing the MAC16 tumour has been shown to be due to a depression of up to 60% in protein synthesis accompanied by a 240% increase in protein degradation [14]. In the present study, leucine has been shown to attenuate both the increase in phosphorylation of PKR and eIF2α in skeletal muscle of mice bearing the MAC16 tumour, as well as in murine myotubes exposed to PIF. The concentration of leucine employed in vitro (2 mmol/l) is similar to that reported previously [8] in serum of rats when leucine was administered at 1.35 g/kg of body weight. The effect of leucine on the phosphorylation of PKR appears to arise from an increase in the expression of PP1, which has been shown to bind to the N-terminal regulatory region of PKR and inhibit autophosphorylation [19]. The ability of leucine to attenuate activation of PKR would explain, at least in part, the ability to increase protein synthesis in skeletal muscle and attenuate protein degradation, as shown from in vitro experiments with PIF. Inhibition of PKR may also explain the small, but significant, inhibition of tumour growth by leucine, since inhibition of PKR by a low-molecular-mass inhibitor also inhibits tumour growth [18].

Stimulation of protein synthesis in the skeletal muscle of cachetic mice by leucine is also due to increases in the eIF4F complex, which promotes the migration and recruitment of the 43S pre-initiation complex to the mRNA, enhancing peptide-chain initiation [23]. The eIF4F complex is made up of three subunits: eIF4E, eIF4G and eIF4A, with eIF4E being considered to be rate-limiting in binding on mRNA to ribosomes, since it is the least abundant in muscle [24]. The assembly of the eIF4E complex is in part controlled by 4E-BP1, which sequesters eIF4E, preventing it from interacting with eIF4G [24]. Phosphorylation of 4E-BP1 in response to growth factors, or BCAAs, results in the release of eIF4E from the inactive 4E-BP1–eIF4E complex, allowing it to interact with eIF4G [9]. In the gastrocnemius muscle of weight-losing mice bearing the MAC16 tumour, there was a reduction in the phosphorylation of 4E-BP1 with increasing weight loss, resulting in an increase in the amount of eIF4E associated with 4E-BP1, and a decrease in the levels associated with eIF4G. The activity of eIF4E can also be regulated by phosphorylation, with the phosphorylated form having an increased affinity for the cap structure [25]; however, phosphorylation of eIF4E was not as affected by weight loss as it was by cellular stress [26]. Treatment of weight-losing mice with leucine attenuated the depression in protein synthesis, and this was associated with an increase in phosphorylation of 4E-BP1, resulting in a reduced binding of eIF4E with 4E-BP1, and an increased association with eIF4G. Recent results [27] show an increased expression of eIF4E and eIF4G in the gastrocnemius.
muscle of pregnant rats bearing the Walker 256 tumour and treated with a leucine-rich diet.

The decreased levels of the eIF4E complex with increasing weight loss may be due to decreases in plasma insulin levels [11], since insulin has been shown to stimulate phosphorylation of 4E-BP1, decreasing its association with eIF4E and increasing the active eIF4F complex [28]. Changes in 4E-BP1 phosphorylation modulated by insulin and leucine occur through the mTOR pathway, which also results in activation of p70S6K [29]. Phosphorylation of p70S6K is associated with its activation causing selective translation of mRNAs that contain a 5′-polypyrimidine tract, which codes for proteins that are generally involved in the translation apparatus [30]. Levels of both phosphorylated forms of p70S6K and mTOR in skeletal muscle of mice bearing the MAC16 tumour were found to decrease with increasing weight loss, and these were restored to values found in mice without weight loss after treatment with leucine. Previous studies in food-deprived rats have shown that leucine stimulates phosphorylation of p70S6K independently of increases in serum insulin [9]. Although mTOR can directly phosphorylate the N-terminal threonine sites in 4E-BP1 in vitro, the effect is most likely to arise from repression of the inhibitory action of TSC1–TSC2 (where TSC is tuberous sclerosis complex) on mTOR signalling [31].

Increasing weight loss was also associated with an increased phosphorylation of the elongation factor eEF2, which would result in an inhibition of elongation by decreasing its affinity for the ribosome 10–100 times [32]. This would reduce global protein synthesis. Treatment of cachectic mice with leucine decreased the phosphorylation of eEF2 in gastrocnemius muscle to levels found in animals without weight loss, and this would attenuate the depression of protein synthesis. Leucine starvation has been shown previously [33] to increase phosphorylation of eEF2 in murine myotubes, whereas insulin induces dephosphorylation of eEF2 via mTOR [34]. Since leucine has been shown to stimulate the mTOR pathway, it may act by a similar mechanism.

Some of the effects of leucine on protein synthesis in vivo may be due to its ability to transiently stimulate insulin secretion [35]. Both increased leucine and insulin are required for sustained stimulation of protein synthesis in skeletal muscle of food-deprived rats. Somatostatin, an inhibitor of pancreatic hormone release, prevented the leucine-induced changes in serum insulin
and protein synthesis and attenuated the leucine-induced changes in the 4E-BP1 and p70S6K phosphorylation in skeletal muscle. However, it had no effect on eIF4G–eIF4E assembly, which by itself is insufficient to stimulate the rates of protein synthesis in skeletal muscle. Another study [36] found that the rates of protein synthesis in diabetic rats were higher when they were fed leucine than when they remained food-deprived, suggesting that a portion of the protein synthetic response must occur through an insulin-independent pathway and in the absence of changes in phosphorylation of 4E-BP1 and p70S6K. Leucine was also shown to attenuate the depression in protein synthesis and the increase in protein degradation in murine myotubes in response to PIF, which has been attributed to its ability to attenuate activation of PKR and the subsequent phosphorylation of eIF2α, and it is possible that this may be important in the diabetic state. Leucine also stimulated the basal rate of protein synthesis in myotubes, but had no effect on the basal rate of protein degradation, because both are independent of PKR activation in the absence of a stimulus (PIF). Leucine could stimulate the basal rate of protein synthesis through an increase in the eIF4G–eIF4E complex and a reduced phosphorylation of eIF2. Although PKR has been shown to increase expression of the ubiquitin–proteasome proteolytic pathway [2], basal protein degradation has been shown [37] not to occur through this pathway, but through a Ca2+ -dependent proteolytic system (calpains). Thus, although leucine can attenuate the induced (PKR-dependent) protein degradation, it would not be expected to influence the basal rate.

Thus BCAAs, and leucine in particular, have a protective effect on skeletal muscle atrophy in cancer cachexia. Leucine acts to stimulate protein synthesis and reduce protein degradation. The effect on protein synthesis is likely to arise from multiple effects, including dephosphorylation of eIF2α, an increase in eIF4F complex formation, increased activity of p70S6K, and decreased phosphorylation of eIF2. The effect on protein degradation is most likely to arise from attenuation of the phosphorylation of PKR through increased expression of PP1.

This could be employed as a therapeutic regime to treat cachectic cancer patients, since oral supplementation with leucine at much lower levels (0.052 g/kg of body weight) than employed in the murine study have been reported to stimulate muscle protein synthesis in elderly men [38].

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Figure 8 Effect on phosphorylated and total 4E-BP1, eIF4E and eIF2 from non-tumour-bearing mice, and mice bearing the MAC16 tumour treated with either PBS or leucine.

Western blots showing the amount of 4E-BP1 (A) and eIF4E (B) associated with eIF4E, and phosphorylation states of 4E-BP1 (p 4E-BP1) (C), eIF4E (p eIF4E) (D) and eIF2 (p eIF2) (E) in gastrocnemius muscle of non-tumour-bearing mice (NTB), and in mice bearing the MAC16 tumour treated with PBS (MAC16) or leucine (MAC16 + Leu). The details of the experiment are given in the Experimental section and in the legend to Figure 5. Samples were taken at the end of the experiment depicted in Figure 5. Representative Western blots are shown, and the densitometric analysis, shown underneath, represents the means ± S.E.M. for three separate Western blots. The ratio of phospho (ph) to total (tot) forms is shown in (F). *P < 0.05 and †P < 0.01 compared with non-tumour-bearing animals; ††P < 0.05 and †††P < 0.01 compared with mice bearing the MAC16 tumour treated with PBS.


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