Effect of eicosapentaenoic acid, protein and amino acids on protein synthesis and degradation in skeletal muscle of cachectic mice

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Atrophy of skeletal muscle reduces both the quality and quantity of life of patients with cancer cachexia. Loss of muscle mass is thought to arise from a reduction in protein synthesis combined with an enhanced rate of protein degradation, and few treatments are available to counteract this process. Eicosapentaenoic acid (EPA) has been shown to attenuate the enhanced protein degradation, but to have no effect on protein synthesis. This study examines the effect of EPA combined with a protein and amino-acid supplementation on protein synthesis and degradation in gastrocnemius muscle of mice bearing the cachexia-inducing MAC16 tumour. Muscles from cachectic mice showed an 80% reduction in protein synthesis and about a 50-fold increase in protein degradation compared with muscles from nontumour-bearing mice of the same age and weight. Treatment with EPA ($1\,g\,kg^{-1}$) daily reduced protein degradation by 88%, but had no effect on protein synthesis. Combination of EPA with casein ($5.35\,g\,kg^{-1}$) also had no effect on protein synthesis, but when combined with the amino acids leucine, arginine and methionine there was almost a doubling of protein synthesis. The addition of carbohydrate ($10.7\,g\,kg^{-1}$) to stimulate insulin release had no additional effect. The combination involving the amino acids produced almost a doubling of the ratio of protein synthesis to protein degradation in gastrocnemius muscle over that of EPA alone. No treatment had a significant effect on tumour growth rate, but the inclusion of amino acids had a more significant effect on weight loss induced by the MAC16 tumour than that of EPA alone. The results suggest that combination therapy of cancer cachexia involving both inhibition of the enhanced protein degradation and stimulation of the reduced protein synthesis may be more effective than either treatment alone.

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Atrophy of skeletal muscle in cancer cachexia results in weakness (asthenia) and death. Muscle mass is controlled by the rate of protein synthesis and the rate of protein degradation, which in the adult are normally in balance, so that muscle mass remains constant. In cachexia whole body protein turnover is increased (Carmichael *et al*, 1980), while in skeletal muscle protein synthesis is decreased (Lundholm *et al*, 1976), while protein degradation is elevated (Lundholm *et al*, 1982). Attempts to increase protein synthesis, for example, by elevation of substrate flux, in the absence of inhibitors of protein degradation have not increased lean body mass (Evans *et al*, 1985). Protein degradation in cancer cachexia has been attributed predominantly to an increased expression of the ubiquitin–proteasome proteolytic pathway (Lecker *et al*, 1999). One of the few agents capable of attenuating protein degradation in cachexia is the polyunsaturated fatty acid, eicosapentaenoic acid (EPA), which downregulates the increased gene expression of key regulatory components of the ubiquitin–proteasome pathway in skeletal muscle (Whitehouse *et al*, 2001). Although EPA attenuates the increased protein degradation in cancer cachexia, it has no effect on the depression of protein synthesis (Beck *et al*, 1991). Thus, EPA attenuates the development of weight loss in cachectic patients with pancreatic cancer, but weight gain is minimal (Wigmore *et al*, 2000). However, when patients were administered EPA together with a high-protein high-energy supplement weight gain was seen and this arose solely from an increase in lean body mass (Barber *et al*, 1999).

This suggests a mechanism to counter muscle atrophy in cancer cachexia by the inhibition of protein degradation, combined with a stimulus for protein synthesis. Muscle protein synthesis has been shown to be initiated by essential amino acids, particularly the branched chain amino-acid (BCAA) leucine (Anthony *et al*, 2000). Stimulation of protein synthesis requires an optimal plasma profile of amino acids and occurs through a mechanism that involves the stimulation of the eukaryotic initiation factor (eIF)-binding protein 1. Arginine is a conditionally essential amino acid, which has been shown to be deficient in tumour-bearing mice (Vissers *et al*, 2002). Arginine may improve the delivery of amino acids to muscle (Robertson *et al*, 1994), and thus may increase protein synthesis. Methionine is another essential amino acid, which may

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be rate limiting in protein synthesis. It has recently been shown (Tesseraud *et al*, 2003) that simultaneous addition of two amino acids, leucine and methionine, are critical in controlling protein translation. Methionine is not only a building block for proteins but is also the primary amino acid needed to initiate protein synthesis (Bradshaw *et al*, 1998). In addition, synthesis of acute phase proteins, which has been correlated with the loss of body mass in patients with lung and gastrointestinal cancers (McMillan *et al*, 1998), alters requirements for amino acids, since these proteins contain relatively high levels of sulphur-containing amino acids (Reeds *et al*, 1997). Lower plasma levels of leucine and methionine have been reported in the serum of weight-losing mice bearing the MAC16 tumour (Beck and Tisdale, 1989).

The purpose of the present study was to investigate the effect of protein (casein) and amino acids (leucine, arginine and methionine) together with EPA on protein synthesis and degradation in gastrocnemius muscle of mice bearing the MAC16 colon adenocarcinoma (Bibby *et al*, 1987). An additional group received carbohydrate, to stimulate insulin release, which should facilitate the uptake of amino acids into muscle and stimulate protein synthesis.

MATERIALS AND METHODS

Animals

Pure strain male NMRI mice were bred in our own colony and fed economy rodent breeder diet (Special Diet Services, Essex, UK) and water *ad libitum*. The diet contained 19.2% crude protein and 4.3% fat. Animals were implanted subcutaneously (s.c.) in the flank with fragments of the MAC16 tumour by means of a trochar, selecting from donor animals with established weight loss (Bibby *et al*, 1987). All animal experiments followed a strict protocol, approved by the British Home Office, and the ethical guidelines that were followed meet the standards required by the UKCCR guidelines (Workman *et al*, 1998). Weight loss was evident 10–12 days after transplantation and the animals were randomised into groups of 12 when the weight loss was about 5%. Six animals were used for protein synthesis and six for protein degradation. A nontumour-bearing group formed an additional control. Cachectic mice received olive oil as a control, EPA ($1.0 \, g \, kg^{-1}$), EPA ($1.0 \, g \, kg^{-1}$ and casein protein $5.35 \, g \, kg^{-1}$), EPA ($1.0 \, g \, kg^{-1}$, casein $5.35 \, g \, kg^{-1}$, leucine 25.5 mg, arginine 12 mg, methionine 5 mg) and EPA ($1.0 \, g \, kg^{-1}$, casein $5.35 \, g \, kg^{-1}$, amino acids as above and $10.7 \, g \, kg^{-1}$ carbohydrate). The supplements were administered twice daily in $50 \, \mu l$ doses. All treatments were administered daily peritoneally (p.o.) by gavage for a 4-day period and protein synthesis and degradation were determined on the 4th day. The concentrations of protein, amino acids and carbohydrate were equivalent to those consumed by patients taking the recommended dosage of Resource® Support (Novartis Medical Nutrition). Tumour volumes were measured daily by means of calipers, and were recorded as a percentage of the starting tumour volume. Body weight was measured daily and recorded as the change in body weight from the start of the experiment. At the end of the experiment, the animals were humanely killed and the gastrocnemius muscles were rapidly removed for further analysis.

Materials

L-[4^{-3}H]Phenylalanine (specific activity $30 \, Ci \, mmol^{-1}$) was purchased from Amersham Life Science Products, Amersham UK. EPA as the synthetic triglyceride, Omegavie 90, contained 93.2% EPA as the fatty acid, and was purchased from Polaris, France. The carbohydrate was 10DE maltodextrin from Grain Processing Corporation, USA. The protein was a casein hydrolysate with the same amino-acid profile as casein and was purchased from New Zealand Milk Products.

Protein synthesis and degradation in gastrocnemius muscle

The method for the determination of protein synthesis and degradation in gastrocnemius muscle has been described previously (Beck *et al*, 1991). Briefly, protein synthesis was measured by the incorporation of L-[4^{-3}H]phenylalanine during a 2 h period in which isolated gastrocnemius muscles were incubated at 37°C in RPMI 1640 without phenol red and saturated with $O_2 : CO_2$ (19:1). After incubation, muscles were rinsed in nonradioactive medium, blotted and homogenised in 4 ml 2% 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 assays animals from the same group as used to measure protein synthesis were administered i.p. with $0.4 \, mM$ L-[4^{-3}H]phenylalanine in phosphate-buffered saline ($100 \, \mu l$) 24 h prior to the assay. Isolated gastrocnemius muscles were extensively washed with 0.9% NaCl and RPMI 1640 medium before measuring the release of radioactivity into RPMI 1640 over a 2 h period. The protein-bound radioactivity was determined by homogenising in 2% perchloric acid as above and the rate of protein degradation was calculated by dividing the amount of [^{3}H]phenylalanine radioactivity released into the incubation medium during the 2 h incubation period by the specific activity of protein-bound [^{3}H]phenylalanine.

Statistical analysis

Results are expressed as mean $\pm$ s.e.m. Differences were determined by one-way analysis of variance (ANOVA) followed by Tukey–Kramer multiple comparison test. *P*-values less than 0.05 were considered significant.

RESULTS

The effects of the various treatments on protein synthesis and protein degradation in gastrocnemius muscle of cachectic mice bearing the MAC16 tumour is shown in Figure 1. The concentration of EPA used was suboptimal in order to discern synergistic interactions. Control animals showed high levels of protein synthesis and little protein degradation, which was expected, since they were growing steadily. There was a significant depression in protein synthesis and a large increase in protein degradation in muscles of cachectic mice bearing the MAC16 tumour, as reported previously (Beck *et al*, 1991). EPA treatment significantly reduced protein degradation, but had no effect on protein synthesis. There was no significant effect of any of the other treatments on protein degradation compared with that of EPA alone. There was a small, but nonsignificant elevation in protein synthesis in animals receiving casein in addition to EPA. However, when casein was administered together with leucine, arginine and methionine, there was a significant increase in protein synthesis above that seen in cachectic mice or cachectic mice administered EPA. Interestingly, this effect was not seen in the presence of carbohydrate. The ratio of protein synthesis to degradation was improved by all supplement treatments over that of EPA alone (Figure 2), although it never reached the values observed in nontumour-bearing mice.

The effect of the various treatments on body weight loss is shown in Figure 3. Both EPA and EPA + casein reduced total body weight loss, but this did not reach significance except at the 4-day time point. However, the addition of both amino acids and amino acids + carbohydrate to the EPA + casein regime caused a significant reduction in weight loss compared with nontreated controls. None of the treatments had a significant effect on tumour volume (Figure 4).
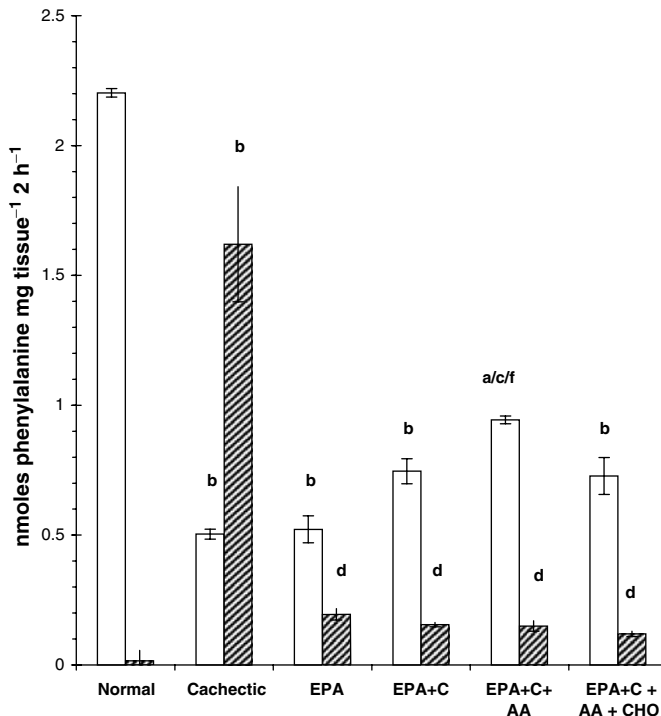
Figure 1 Protein synthesis (□) and degradation (▨) in gastrocnemius muscle of nontumour-bearing mice (normal), cachectic mice (cachectic), cachectic mice treated with EPA (EPA), cachectic mice treated with EPA + casein (EPA + C), cachectic mice treated with EPA + casein + amino acids (EPA + C + AA), cachectic mice treated with EPA + casein + amino acids + carbohydrate (EPA + C + AA + CHO). The details of the treatments are given in the Materials and methods section. The weight loss in the various groups is shown in Figure 3. Differences from control are indicated as a, $P < 0.01$ and b, $P < 0.001$, while differences from cachectic are indicated as c, $P < 0.05$ and d, $P < 0.001$ and differences from EPA as f, $P < 0.05$. The number of animals used for both protein synthesis and protein degradation $n = 6$.
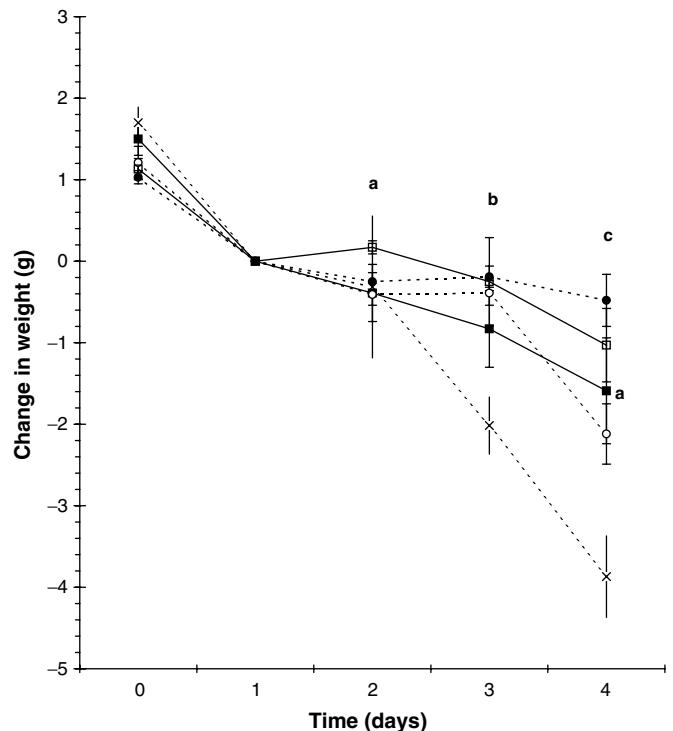

Figure 3 Effect of nutritional supplementation on body weight loss in mice bearing the MAC16 tumour. All animals were weight losing at the time of initiation of the experiment (day 1). Animals ($n = 12$) were randomised to receive daily p.o. treatments of olive oil (X), EPA (■), EPA + casein (○), EPA + casein + amino acids (●) or EPA + casein + amino acids + carbohydrate (□) at the concentrations indicated in the Materials and methods section. Differences from animals receiving olive oil alone are indicated as a, $P < 0.05$, b, $P < 0.01$ and c, $P < 0.001$.
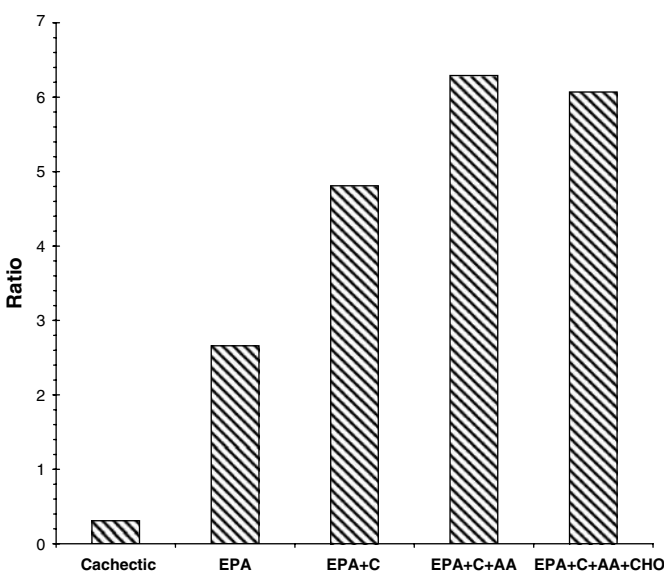

Figure 2 Ratio of protein synthesis to protein degradation in gastrocnemius muscle of cachectic mice bearing the MAC16 tumour after being subjected to the nutritional regimes detailed in Figure 1 for a 4-day period.
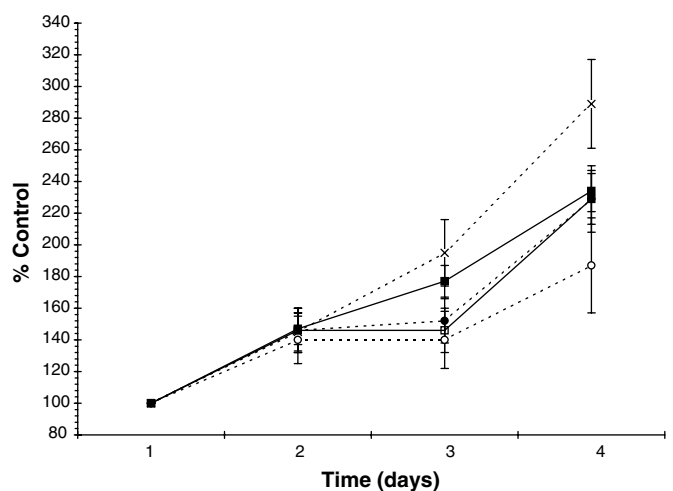

Figure 4 Effect of nutritional supplementation on tumour growth in mice bearing the MAC16 tumour. Tumour size has been normalised to 100% at the start of the experiment. Animals ($n = 12$) were randomised to receive daily p.o. treatments of olive oil (X), EPA (■), EPA + casein (○), EPA + casein + amino acids (●) or EPA + casein + amino acids + carbohydrate (□) at the concentrations indicated in the Materials and methods section. There was no significant difference between the groups.

DISCUSSION

The mechanism for the increased protein degradation in skeletal muscle in cancer cachexia has been suggested as an increased expression of the ubiquitin–proteasome proteolytic pathway (Williams et al, 1999). Various cytokines, such as tumour necrosis factor-α or interferon gamma (Llovera et al, 1998), have been shown to increase transcripts of ubiquitin in skeletal muscle, but the importance of these cytokines as mediators of the weight loss in human cancer cachexia is controversial (Maltoni et al, 1997). In cancer cachexia, a sulphated glycoprotein, proteolysis-inducing factor (PIF) produced by cachexia inducing tumours (Todorov et al, 1996) may be more important in skeletal muscle atrophy. Proteolysis-inducing factor stimulates loss of the myofibrillar protein myosin directly by increasing the expression of proteasome subunits and the ubiquitin-conjugating enzyme ($E2_{14k}$) (Gomes-Marcondes et al, 2002). The process is initiated by the activation of PIF of an intracellular signalling cascade in muscle involving the release of arachidonic acid from membrane phospholipids and metabolism to 15-hydroxyeicosatetraenoic acid (15-HETE) (Smith et al, 1999), and involving the activation of the transcription factor nuclear factor-κB (NF-κB) (Whitehouse and Tisdale, 2003). Eicosapentaenoic acid attenuates this process, both by inhibiting the formation of 15-HETE (Smith et al, 1999) and by stabilising the IκB/NF-κB cytosolic complex and nuclear accumulation of NF-κB (Whitehouse and Tisdale, 2003), preventing the increase in proteasome expression and degradation of myofibrillar proteins. Proteasome-mediated proteolysis is independent of the amount of protein consumed, so that simple nutritional supplementation would not be expected to prevent muscle catabolism as observed in mice bearing the MAC16 tumour. However, leucine has been shown to inhibit the expression of genes of the proteasome pathway in muscle of cachectic rats, but not in those of rats after starvation (Busquets et al, 2002). Branched chain amino acids also directly inhibit the proteasome 'chymotrypsin-like' enzyme activity, the predominant proteolytic activity of the β-subunits of the proteasome in skeletal muscle, but not in the liver (Hamel et al, 2003). Despite this in the present study, there was no

evidence for attenuation of protein degradation by leucine/amino-acid mixture below that induced by EPA alone in gastrocnemius muscle of cachectic mice bearing the MAC16 tumour.

In addition to the effect on protein degradation PIF also inhibits protein synthesis in muscle, but the mechanism by which this occurs is not known, although it is attenuated by insulin at physiological concentrations and below, but not by EPA (Smith et al, 1999). The action of insulin suggests that PIF may inhibit translation, for example, by dephosphorylation of translation factors or the S6 ribosomal protein. Branched chain amino-acid, and leucine in particular, enhance muscle protein synthesis through the activation of the mRNA-binding step in translation initiation in skeletal muscle, but not in the liver (Yoshizawa, 2004). The action of leucine is mediated by the activation of the ribosomal protein S6 kinase and by phosphorylation of initiation factors, suggesting that it may, like insulin, overcome the PIF-induced inhibition of protein synthesis. This study shows that while casein alone did not significantly stimulate protein synthesis in gastrocnemius muscle of cachectic mice, there was a significant increase in protein synthesis with the addition of the leucine/arginine/methionine mix. This was not further enhanced by the carbohydrate mix, although leucine and arginine have been previously shown to stimulate insulin secretion (Malaisse, 1984), and insulin plays a permissive role in leucine-induced protein synthesis (Yoshizawa, 2004). Thus treatment of cancer patients with a combination of EPA, protein and amino acids, particularly leucine, would be expected to attenuate protein degradation and stimulate protein synthesis in skeletal muscle, which should lead to improvements both in the quality of life and survival, and this combination is currently under clinical evaluation.

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