Proteasome activity is altered in skeletal muscle tissue of tumour-bearing rats fed a leucine-rich diet

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Abstract

Leucine can modulate skeletal muscle metabolism by enhancing protein synthesis and decreasing proteolysis. In this study, we investigated the effects of leucine on the ubiquitin–proteasome system in skeletal muscle of pregnant tumour-bearing rats fed a leucine-rich diet. Pregnant Wistar rats were distributed into three groups that were fed a semi-purified control diet (C, control; W, Walker tumour-bearing; P, pair-fed) and three other groups of pregnant rats fed a semi-purified leucine-rich diet (L, leucine; WL, Walker tumour-bearing; PL, pair-fed). The tumour-bearing rats were injected subcutaneously with a suspension of Walker 256 tumour cells. Protein synthesis and degradation were measured in gastrocnemius muscle; the total protein content and tissue chymotrypsin-like and alkaline phosphatase enzyme activities were also determined. Muscle protein extracts were run on SDS-PAGE to assess the expression of the myosin heavy chain (MHC), 20S α proteasome subunit, 19S MSSI ATPase regulator subunit and 11S α subunit. Although tumour growth decreased the incorporation of [3 H]-Phe, the concomitant feeding of a leucine-rich diet increased the rate of protein synthesis. Muscle proteolysis in both tumour-bearing groups was increased more than in the respective control groups. Conversely, the leucine-rich diet caused less protein breakdown in the WL group than in the W group. Only the W group showed a significant reduction (71%) in the myosin content. In WL rats, the 20S proteasome content (32 kDa band) was reduced, while the expression of the 19S subunit was 3-fold less than in the W group and the 11S proteasome subunit reduced, to around 32% less than in the W group. These findings clearly indicate that leucine can stimulate protein synthesis and inhibit protein breakdown in pregnant rats, probably by modulating the activation of the ubiquitin–proteasome system during tumour growth.

Endocrine-Related Cancer (2004) 11 887–895

Introduction

Cachexia occurs in about one-half of all cancer patients (Tisdale 1997) and is the major cause of cancer morbidity and mortality. Cachexia is characterised by a progressive loss of body weight, especially in adipose and muscle tissues, which impairs normal functioning. The loss of skeletal muscle in most cachectic patients and animals involves a decrease in protein synthesis and increased protein degradation, as indicated by a variety of metabolic alterations (Ventrucchi *et al.* 2002, Gomes-Marcondes *et al.* 2003).

Muscle proteolysis involves lysosomal and non-lysosomal pathways. Non-lysosomal proteolysis is controlled

by the ubiquitin–proteasome system and involves intracellular and myofibrillar protein breakdown (Kee *et al.* 2002). The muscle ubiquitin pathway is a complex process activated during severe catabolism such as occurs in cancer cachexia (Attaix *et al.* 1999, Wray *et al.* 2002). This process involves multiple steps that are proteasome dependent, including protein ubiquitination mediated by the ubiquitin enzymes E1, E2 and E3 and the 26S proteasome (Attaix *et al.* 2001), which depends on ATP as an energy source. The 26S proteasome is a proteolytic complex that consists of a central core particle, the 20S proteasome subunit, and two regulatory complexes (19S and 11S) that are bound to the end of the 20S subunit (Glickman & Ciechanover 2002).

Leucine plays an important role in skeletal muscle metabolism and can enhance protein synthesis and decrease proteolysis, independently of other branched-chain amino acids (BCAAs) such as isoleucine and valine (Carbo *et al.* 1996a, Anthony *et al.* 2001). Leucine regulates protein synthesis in skeletal muscle following food intake (Anthony *et al.* 2002b). A leucine-rich diet can improve the carcass nitrogen and lean body mass in rats with Walker 256 tumours and in mice with MAC 16 adenocarcinoma (Tisdale 2000, Ventrucci *et al.* 2001). The importance of insulin and amino acids, mainly BCAAs, in regulating protein synthesis has been studied in several situations (Fulks *et al.* 1975). The deleterious effects of tumours are more pronounced when associated with pregnancy since maternal nutrition supplies glucose and amino acids for tumour and foetal development (Carbo *et al.* 1996a) and can result in muscle-wasting proteolysis. In this study, we investigated the effects of leucine on skeletal muscle protein synthesis and degradation, and the activity of the ubiquitin–proteasome system in pregnant tumour-bearing rats fed a leucine-rich diet.

Methods

Animals and diets

Young female Wistar rats (45 days old, $n = 60$) were obtained from the animal facilities of the State University of Campinas, São Paulo, Brazil. Female rats were housed overnight with adult males (four females:one male), according to harem methodology (Baker 1991), and the first day of pregnancy was determined based on the detection of sperm in the vaginal smear. All rats were housed in collective cages under standard conditions ($22 \pm 2^\circ\text{C}$, 12 light:12 h darkness cycle, with free access to water and food). Semi-purified diets were used and consisted of a balanced control diet (C; 18% protein, AIN-93G) and a leucine-rich diet (L; 15% protein with 3% leucine). The diets contained the same amount of carbohydrate (63%), fat (7%) and fibre (5%), in accordance with the AIN-93G (Reeves 1993). The corn standard and dextrin were supplied by Corn Products Brazil Ingredients, the vitamin mix was from DSM Nutritional Products (DSM Produtos Nutricionais Brasil Ltda), and the amino acids were from Ajinomoto Interamericana Ind. & Com. Ltda, Brazil.

The rats were randomly allocated to six groups. Three groups were fed the control diet: C, pregnant; W, pregnant tumour-bearing; P, pregnant pair-fed, this group received the same amount of food as ingested by the W group. Three other pregnant groups were fed with the leucine-rich diet: L, pregnant; WL, pregnant tumour-bearing; PL, pregnant pair-fed, this group received the same amount of food as ingested by the WL group.

Tumour implantation

The rats in groups W and WL received a subcutaneous injection of Walker 256 tumour cells (approximately 0.25×10^6 cells in 0.5 ml saline solution) in the right flank immediately after the confirmation of pregnancy. The other pregnant groups without tumours (controls) received a single injection of 0.5 ml 0.9% (w/v) NaCl in the same region as a control manipulation. All of the groups were monitored for 20 days after tumour implantation. The general guidelines UKCCCR (United Kingdom Coordinating Committee on Cancer Research 1988) for animal welfare were followed, and the protocols were approved by the institutional Committee for Ethics in Animal Research (CEEA-IB/UNICAMP, protocol 217-5).

Experimental procedures

Protein synthesis was assayed in right gastrocnemius muscles, which were weighed and placed in Krebs–Henseleit bicarbonate (KHB) buffer (110 mM NaCl, 25 mM NaHCO_3 , 3.4 mM KCl, 1 mM CaCl_2 , 1 mM MgSO_4 and 1 mM KH_2PO_4 , pH 7.4) supplemented with 5.5 mM glucose and 0.01% (w/v) albumin. The muscles were pre-incubated for 30 min at 37°C with continuous gassing (95% O_2 –5% CO_2) and shaking, as described by Vary *et al.* (1998). After this period, new KHB buffer supplemented with $5 \mu\text{Ci}$ L-[^3H]phenylalanine/ml (Amersham) was added and the incubation continued for a further 2 h. At the end of this period, the muscles were homogenised in 10% trichloroacetic acid (TCA, 1:3 w/v), centrifuged at $10\,000g$ for 15 min at 4°C , and the pellet then suspended in 1 M NaOH and incubated at 40°C for 30 min. Aliquots of this mixture were used to measure the total protein content (Bradford 1976) and to quantify the radioactivity based on liquid scintillation counting of β emissions. The rate of protein synthesis was calculated by the amount of radioactive phenylalanine incorporated in a 2 h period, and was expressed as nanomoles of [^3H]-Phe per microgram of muscle protein (Vary *et al.* 1998).

Protein degradation was assessed using left gastrocnemius muscles which were excised and placed in RPMI 1640 medium and pre-incubated for 30 min under the same conditions of temperature and gas as described above (Vary *et al.* 1998). After the initial incubation, the solutions were replaced by KHB buffer supplemented with cycloheximide (130 $\mu\text{g}/\text{ml}$) followed by a 2 h incubation. At the end of this period, the muscles were dried, weighed and frozen in liquid nitrogen. The muscles and incubation medium were stored at -80°C until analysis. The rate of protein degradation was determined as nanomoles of tyrosine released per microgram of muscle protein per hour, based on fluorometric assay as described by Waalkes & Udenfriend (1957).

Enzymatic activities

The proteins of the gastrocnemius muscle were homogenised in homogenising buffer (HB) (20 mM Tris, 1 mM dithiothreitol, 2 mM ATP and 5 mM MgCl₂) and centrifuged at 15 000 *g* for 15 min at 4 °C. The resulting supernatant was analysed for total protein content (Bradford 1976) and chymotrypsin-like and alkaline phosphatase activities. Chymotrypsin-like activity was determined using the fluorogenic substrate succinyl-Leu-Leu-Val-Try-7-amino-4-methylcoumarin (Suc LLVY-AMC; 0.167 µg/µl in 100 mM Tris-HCl, pH 7.4; excitation 360 nm, emission 460 nm). The activity was expressed as units of fluorescence per microgram of protein, as a percentage of the control group. Alkaline phosphatase activity was measured using 37 mM *p*-nitrophenyl phosphate (PNPP) as substrate, and activity was expressed in nanomoles of nitrophenol formed per microgram of muscle protein (Martins *et al.* 2001).

Myosin and proteasome system

Skeletal muscle proteins were resolved by SDS-PAGE on 12% gels followed by Western blotting. The content of myosin heavy chain (MHC) isoforms was assessed using antibody against MHC at a dilution of 1:250 (Novocastra, Newcastle, UK), followed by detection with a secondary anti-mouse horse radish peroxidase (HRP) antibody (Dako, Carpinteria, USA). The proteasome subunits were analysed by probing 0.45 µm enhanced chemical luminescence (ECL) nylon membranes with antibodies against the 20S α proteasome subunit, the 19S MSSI ATPase regulator subunit and the 11S α subunit (all from Affinity, Newcastle, UK, and diluted 1:1500) followed by detection with the rat anti-mouse secondary HRP-labelled antibody. Actin was used as the loading control, after probing the mouse actin antibody. Images of the gels were captured (FTI 500Image Master VDS, Pharmacia Biotech) and densitometric analyses of the bands were done with Gel Pro Analyser software (Media Cybernetics, Silver Spring, MD, USA).

Statistical analysis

The results are expressed as the means $\pm$ S.E.M. Statistical comparisons were done with one-way ANOVA (Gad & Weil 1994) followed by Bonferroni's test for comparison among groups (Graph Pad Prism software, v3.00 for Windows 98, USA). Statistical significance was considered as a *P* value below 5%.

Results

Protein synthesis

The rate of incorporation of [³H]-Phe by the gastrocnemius muscle, indicative of protein synthesis, was higher in the

L group (around 23.4%) than in the corresponding control group, C (Fig. 1A). Tumour growth reduced this parameter in both tumour-bearing groups (W and WL), with a decrease in [³H]-Phe incorporation by about 47 and 40% respectively, compared with the respective C and L groups. Whereas tumour growth induced severe damage in both pregnant groups (W and WL), the leucine-rich diet fed concomitantly with tumour growth resulted in increased protein synthesis in the WL group (around 1.4-fold higher than in the W group). These results suggest that leucine probably had a protective effect on skeletal muscle

Figure 1 Effects of a leucine-rich diet on protein synthesis (A) and degradation (B) in gastrocnemius muscle from pregnant tumour-bearing rats. Right gastrocnemius muscles from all experimental groups were incubated with [³H]-Phe for 2 h and protein synthesis was then assessed based on the incorporation of radiolabelled amino acid. The contralateral gastrocnemius muscles were similarly incubated for 2 h with cycloheximide to measure protein breakdown based on the release of tyrosine. At least eight to ten rats were used per group. Abbreviations: C, control; W, tumour-bearing rats; P, pair-fed tumour-bearing rats; L, leucine-rich diet group; WL, tumour-bearing rats receiving leucine-rich diet; PL, pair-fed tumour-bearing group fed with a leucine rich-diet. Columns with different letters above are significantly different (*P* < 0.05).

metabolism. Anthony *et al.* (2002b) showed that the oral administration of leucine increased muscle protein synthesis in rats. Pair-fed nutrition does not decrease the rate of protein synthesis, in contrast to the decrease caused by neoplastic growth (Smith & Tisdale 1993). Indeed as shown here, the incorporation of phenylalanine was not reduced in the P and PL groups as compared with the tumour-bearing groups.

Protein degradation

Muscle proteolysis, represented by the release of tyrosine from gastrocnemius muscles, was compared between control and leucine-rich diet groups (Fig. 1B). There was no difference in tyrosine release from skeletal muscle in the C and L groups. However, during tumour growth, there was an imbalance between the rates of protein synthesis and degradation, especially in the W groups. Body weight loss and muscle fatigue are common in cancer cachexia and in women with gynaecological cancers (Olt 2003). In recent studies, we observed a body weight loss and a decrease in total body protein in pregnant rats with Walker 256 tumour (Gomes-Marcendes *et al.* 1998, Ventrucci *et al.* 2001). As shown in Fig. 1B, both tumour-bearing groups showed increased tyrosine release, around 33.5% higher than in the respective control groups, indicating intense muscle protein mobilisation during tumour growth. The tumour-bearing rats fed a leucine-rich diet (WL) showed significantly reduced tyrosine release rates, by around 11% when compared with the W group. An increase in tyrosine release was also verified in the pair-fed groups, P (18%) and PL (21%), when compared with the respective control groups (C and L). However, in the pair-fed situation, the protein breakdown was less intense than in the W and WL groups.

Enzyme activities

The chymotrypsin-like activity (Fig. 2A) was increased in all experimental groups compared with group C, although the activity was lower in the WL group (around 17.8% less) compared with the W group. In the leucine pair-fed group, muscle chymotrypsin-like activity was also similar to the L group. In addition, as presented in Fig. 2B, there was a significant increase in muscle alkaline phosphatase activity only in the pair-fed groups, P and PL, compared with the C and L groups respectively.

MHC in gastrocnemius muscle

Cancer cachexia wastes whole host tissue by specifically reducing the protein carcass mass. Both of the tumour-bearing groups (W and WL) presented a reduction in

Figure 2 Effects of a leucine-rich diet on muscle enzyme activities in tumour-bearing rats. Muscle chymotrypsin-like and alkaline phosphatase activities were measured in gastrocnemius muscle homogenate. Eight to ten rats per group. For abbreviations, see legend to Fig. 1. The columns are the means $\pm$ S.E.M. for eight to ten rats per group. Columns with different letters above are significantly different ($P < 0.05$).

muscle myosin content (Fig. 3). The reduction in group W was significantly greater (71%) than in the corresponding control group (C). On the other hand, there was a significant increase in the myosin content (47%) of gastrocnemius muscle in WL rats in comparison to the W group. Comparatively, as seen in Fig. 3, the muscle myosin content was preserved in both of the pair-fed groups.

Ubiquitin–proteasome system

Muscle proteolysis in cancer cachexia results from high stimulation of the ubiquitin–proteasome system. Feeding a leucine-rich diet to tumour-bearing rats significantly improved the host muscle mass by altering the expression of the proteasome subunits. Both bands of 20S protea-

Figure 3 Effects of a leucine-rich diet on muscle MHC content in tumour-bearing rats. (A) Western blot image of MHC and actin. Actin was used as a loading control. The Western blots are representative data of the best assay from each group (minimum of eight rats per group). (B) Arbitrary densitometric values of myosin expression are analysed for eight individual Western blots. Gastrocnemius muscle homogenates were loaded into SDS-PAGE gels (5 µg protein/well), later transferred to nylon membranes and blotted with an MHC antibody (diluted 1:250). For details see Methods. For abbreviations see the legend to Fig. 1. The columns are the means $\pm$ S.E.M. for eight to ten rats per group. The expression of actin (42 kDa band) showed no difference. Different letters above each column indicate statistical difference ($P < 0.05$).

some subunit (Fig. 4) were significantly increased in the W rats when compared with the other groups. Although the expression levels of the two bands of 20S proteasome slightly increased in groups fed a leucine-rich diet, in the WL group the 20S subunit expression (32 kDa band) was around 32% less than in the W group (W, 0.230 ± 0.012 arbitrary units; WL, 0.156 ± 0.0154 ; $P < 0.05$). There was no significant difference in levels of the 20S subunits between the pair-fed groups. The expression of the 19S subunit (Fig. 5) in the WL group was 3-fold lower than in the W group (W, 0.417 ± 0.017 ; WL, 0.137 ± 0.010 arbitrary units; $P < 0.05$), with no difference in the pair-fed groups. A slight increase in the 11S proteasome subunit (Fig. 6) was observed in the W group compared with group C, but was significantly higher (32% in the W group) only when compared with the pair-fed (P and PL) and the leucine-rich diet groups (W, 0.511 ± 0.068 ; WL, 0.348 ± 0.057 arbitrary units; $P < 0.05$).

Figure 4 Effects of a leucine-rich diet on expression of the 20S α subunit of the 26S proteasome in tumour-bearing rats. (A) Western blot image of 20S subunit and actin. Actin was used as a loading control. Western blots are representative data of the best assay from each group (minimum of eight rats per group). (B) Arbitrary densitometric values of the expression of the 20S subunit (33 and 32 kDa bands) are analysed for eight individual Western blots. Gastrocnemius muscle homogenates were loaded into SDS-PAGE gels (5 µg protein/well), later transferred to nylon membranes and blotted with a 20S α subunit proteasome antibody (diluted 1:1500). For details see Methods. For abbreviations see legend to Fig. 1. The columns are the means $\pm$ S.E.M. for eight to ten rats per group. The expression of actin (42 kDa band) showed no differences. Columns with different letters above are significantly different ($P < 0.05$).

Discussion

The present findings clearly indicate that a leucine-rich diet altered the activation of the ubiquitin–proteasome system during tumour growth. Amino acids, especially

Figure 5 Effects of a leucine-rich diet on expression of the 19S MSSI ATPase subunit of the 26S proteasome in tumour-bearing groups. (A) Western blot image is representative of the best assay from all experimental groups. (B) Arbitrary densitometric values of eight individual Western blots. Gastrocnemius muscle homogenates were loaded into SDS-PAGE gels (5 µg protein/well), later transferred to nylon membranes and blotted with a 19S MSSI ATPase subunit proteasome antibody (diluted 1:1500). For details see Methods. For abbreviations see legend to Fig. 1. The columns are the means $\pm$ S.E.M. for eight to ten rats per group. Columns with different letters above are significantly different ($P < 0.05$).

Figure 6 Effects of a leucine-rich diet on expression of the 11S α subunit of the 26S proteasome in tumour-bearing groups. (A) Western blot image of the 11S α subunit and actin. Actin was used as a loading control. The Western blot is the representative data of the best assay from each group (minimum of eight rats per group). (B) Arbitrary densitometric analysis of 11S α expression values for eight individual Western blots. Gastrocnemius muscle homogenates were loaded into SDS-PAGE gels (5 µg protein/well), later transferred to nylon membranes and blotted with an 11S α subunit proteasome antibody (diluted 1:500). For details see Methods. For the abbreviations see legend to Fig. 1. The expression of actin (42 kDa band) showed no differences. Columns with different letters above are significantly different ($P < 0.05$).

BCAAs, can improve and enhance the rates of protein synthesis in skeletal muscle (Kettelhut *et al.* 1988, Yoshizawa *et al.* 1998, Anthony *et al.* 2002b).

In previous studies, we observed benefits of a leucine-rich diet on carcass mass during Walker tumour growth (Ventrucchi *et al.* 2001, Gomes-Marcondes *et al.* 2003). The present results now show a positive effect of a leucine-rich diet on muscle protein turnover.

Tumour growth causes intense tissue protein turnover by reducing protein synthesis and/or by increasing protein catabolism (Smith & Tisdale 1993, Tessitore *et al.* 1993). Since a reduction in caloric intake does not decrease skeletal muscle mass, as shown in pair-fed rats of the present study and verified by other studies in animals with cachectic tumours (Smith & Tisdale 1993), the cause of the increase in muscle wasting associated with cancer

cachexia remains unknown (Tisdale 2002, 2003). Although Walker 256 tumour growth decreases skeletal muscle and whole maternal body weight, feeding a leucine-rich diet prevented the intense reduction, as previously reported (Ventrucchi *et al.* 2001, Gomes-Marcondes *et al.* 2003). As shown here, 20 days after tumour implantation, a leucine-rich diet significantly enhanced protein synthesis (Fig. 1A). As reviewed by Anthony *et al.* (2001), BCAAs can regulate protein synthesis in skeletal muscle. Protein synthesis is stimulated in starved rats, especially after the infusion of amino acids by total parenteral nutrition (Svanberg *et al.* 1998). These authors also noted that the protein synthesis was enhanced in myofibrillar L6 cell culture, following the addition of amino acids plus insulin-like growth factor (IGF-I) to the culture medium.

The acute administration of leucine stimulates protein synthesis in various tissues of fasted rat (Vary *et al.* 1999, Anthony *et al.* 2002b, Lynch *et al.* 2002). The administration of leucine by gavage to food-deprived rats elevated the rates of protein synthesis in skeletal muscle (Anthony *et al.* 2002a). In rats perfused with amino acid solutions, Vary *et al.* (1999) verified that amino acid supplementation with leucine increased the total protein synthesis in gastrocnemius muscle compared with values obtained with a leucine-poor solution.

Our results support the hypothesis that leucine alone has beneficial effects on skeletal muscle metabolism in tumour-bearing animals, and this was confirmed by the increase in the myosin content (Fig. 3) of the gastrocnemius muscle in WL animals.

In agreement with the idea that leucine alone can improve protein metabolism in tumour-bearing rats, the ingestion of a leucine-rich diet during pregnancy partly inhibited the proteolysis seen in the WL group. Busquets *et al.* (2000) showed that leucine (10 mM solution) inhibited muscle proteolysis in the extensor digitorum and soleus muscles after incubation with a solution to activate the proteolytic system. Recently, Busquets *et al.* (2002) reported that the addition of leucine to the incubation medium had no effect on total muscle proteolysis in muscles from Yoshida tumour-bearing rats. In contrast to Busquets *et al.* (2002), we found that leucine alone stimulated protein synthesis, and inhibited the protein breakdown, probably by reducing the activation of the ubiquitin–proteasome system.

The ATPase subunit of the 19S complex (MSSI) and the 11S subunit associate with the 20S proteasome (Coux *et al.* 1996), and these two regulatory complexes can be responsible for modulated activation of proteolysis; in addition, the two 20S subunits, α and β , can be regulated independently (Attaix *et al.* 1998, Tanahashi *et al.* 1999). An increased expression of the MSSI could facilitate the rapid proteolysis of muscles in Walker tumour-bearing rats since the MSSI unit provides energy for the breakdown of ubiquitinated proteins by the 26S proteasome (Coux *et al.* 1996). The enhanced proteolysis seen in cancer-wasting conditions is ATP dependent (Attaix *et al.* 1997, 1998) since there is an increase in the expression of several ATPases, including MSSI (Dawson *et al.* 1995, Fujita *et al.* 1996, Combaret *et al.* 1999). Since ATPases provide energy for assembly of the 26S proteasome and for the breakdown of ubiquitin conjugates (Attaix *et al.* 1998, Combaret *et al.* 1999), the reduction seen here in the 19S proteasome MSSI subunit parallel to an increase in the myosin content suggested that the ubiquitin–proteasome system may be regulated by BCAAs, such as leucine. Larbaud *et al.* (1996) showed that ubiquitin was also regulated by insulin, with a marked decrease in ubiquitin expression in skeletal muscle

after treatment with insulin. Insulin can improve the host carcass protein turnover (Curi *et al.* 1995, Fernandes *et al.* 1996a,b), and Anthony *et al.* (2002a) have observed simultaneous stimulatory effects of insulin and leucine on protein turnover in skeletal muscle.

In an extensive review, Coux *et al.* (1996) suggested that the 11S regulator subunit associates with the 20S proteasome to stimulate peptidase activity and could be involved in the final breakdown of peptides derived from protein degradation. Although there was no significant difference in 11S expression between the W and C groups, this expression was lower in the WL group, suggesting that supplementation with leucine conferred some advantage.

Muscle protein mobilisation can be an adaptable process to several conditions such as pregnancy in order to provide amino acids and an adequate nutritional supply for foetal growth (Carbo *et al.* 1998); it can also adapt to diseases, to provide amino acids for acute energy metabolism cost and other essential processes for organism survival. On the other hand, tumour growth can affect protein turnover and lead to ineffective amino acid transport between the mother and foetus (Carbo *et al.* 1996b). Since adequate maternal nutrition is fundamental for foetal welfare and placental development (Godfrey 2002), protein catabolism intensifies in late pregnancy to provide enough nutritional support for foetal growth (King 2000). Conversely, progressive tumour development leads to foetal weight loss (Ventrucci *et al.* 2001, 2002) and compromises the placental–foetal exchange (Toledo & Gomes-Marcondes 1999), mainly through the direct and/or indirect effects of cytokines produced by the tumour and/or host cells (Toledo & Gomes-Marcondes 2004a,b). Other studies have shown that cytokines (Kettelhut *et al.* 1988) and, more recently, a proteolysis-inducing factor (Gomes-Marcondes *et al.* 2002, Hasselgren *et al.* 2002, Tisdale 2003) cause intense muscle wasting by activation of the ubiquitin–proteasome system.

The precise mechanism by which leucine and/or BCAAs can induce protein synthesis and/or inhibit protein breakdown needs to be better understood. Investigations are currently underway to address these important questions. In practical terms, our results suggest that the ingestion of a leucine-rich diet could be beneficial in reducing the muscle wasting associated with cancer cachexia.

Acknowledgements

This study was supported by grants from Fundação de Amparo à Pesquisa do Estado de São Paulo (Fapesp)

(96/9463-9466, 01/02135-02133, 02/04464-04467), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (521048/95-98, 522755/96-98, 350047/03-00), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Fundo de Apoio ao Ensino e à Pesquisa (FAEP-UNICAMP).

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