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ORIGINAL ARTICLE

Leucine affects the fibroblastic Vero cells stimulating the cell proliferation and modulating the proteolysis process

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Abstract Branched-chain amino acids, especially leucine, exert regulatory influences on protein and carbohydrate metabolism, ribosome biogenesis and gene expression. This study investigated the effects of leucine in fibroblastic cells analysing viability, proliferation, morphology, proteolysis enzymes activities and protein turnover. After exposure to culture medium enriched with 25 or 50 μM leucine for 24, 48 and 72 h, Vero cells have no alterations on viability and morphology. Leucine-treated cells showed increase on alkaline phosphatase activity and proliferation. The protein synthesis was slightly increased, whereas the protein degradation showed a deep reduction after leucine incubation. The chymotrypsin-like, cathepsin B and H and calpain activities were decreased in leucine-treated cells. In conclusion, the proteolytic pathways and the total protein degradation were modulated by leucine in Vero cells. Our observations support the concept that Vero cells may represent a new model for protein turnover study.

Keywords Leucine · Protein metabolism · Cell proliferation · Vero cells

Introduction

The branched-chain amino acids (BCAA) leucine, isoleucine, and valine are amino acids that cannot be synthesized by animals, are therefore essential nutrients that must be obtained from foods and they are required specifically for the synthesis of proteins (Harper et al. 1984). Leucine and isoleucine are known to be good sources of energy, producing 40 mol ATP/mol in specific tissues (Ichihara 1999). In addition to provide the substrates for protein synthesis or sources of energy, branched-chain amino acids, especially leucine, exert regulatory influences on carbohydrate metabolism (Layman and Baum 2004), translation initiation (Stipanuk 2007), ribosome biogenesis (Anthony et al. 2001) and gene transcription (Kimball and Jefferson 2006). Meanwhile, leucine is a key factor in the modulation of skeletal muscle protein turnover, in vivo (Biolo et al. 2006, Kobayashi et al. 2006) and in vitro (Nakai et al. 2006). Leucine stimulates protein synthesis especially improving the eukaryotic initiation factors expression (Ventrucci et al. 2007), and signalling through the mTOR pathway (Avruch et al. 2008; Proud 2007). In addition, leucine down regulates the proteolysis by inhibiting the ATP-ubiquitin–proteasome pathway (Nakashima et al. 2005; Ventrucci et al. 2004) and inhibiting the lysosomal autophagy also through the mTOR-dependent pathway (May and Buse 1989; Meijer and Codogno 2008). Moreover, Sun and Zemel (2007) have suggested that leucine participates in the regulation of fat metabolism in murine adipocytes and muscle cells in vitro. Additionally, effects of BCAA including induction of hepatocyte DNA synthesis and proliferation (Kimura and Ogihara 2005) and pancreatic β-cells proliferation in vitro (Xu et al. 1998) indicate that leucine can possible act in mitogenic signalling. Conversely, cancer can spoliate the main body sources, as skeletal mass, including the non- and collagenic-nitrogen from body composition (Gomes-Marcondes et al. 2003; Ventrucci et al. 2001). As the leucine-supplemented diet improved the total body and muscle collagenic mass wasted in tumour-bearing rats (Ventrucci et al. 2001), it is worth to investigate how the fibroblast cells can response to

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leucine, as the collagenic nitrogen could be inferred as the fibroblast activity. Knowing that leucine effects have been studied mainly in hepatocytes and muscle cells, in this study we utilized Vero cells, a fibroblastic lineage, which the metabolism could infer the extracellular matrix turnover. The aim of the present work was to investigate the effects of leucine on cell viability, morphology, proliferation, activity of proteolytic systems (ubiquitin–proteasome, lysosomal and calcium-dependent pathways), and protein synthesis and degradation in Vero cells culture.

Materials and methods

Chemicals

The reagents are purchased from Labsynth (Diadema, Brazil); Merck (Darmstadt, Germany); Promega (Madison, USA); Riedel-de Haën (Hanover, Germany), and Sigma Chemical Company (St Louis, USA).

Cell culture

Vero cells, obtained from the Adolfo Lutz Institute (São Paulo, Brazil), at passage 162, were maintained in DMEM (Dulbecco's modified Eagle's medium, Sigma) supplemented with 5% of fetal bovine serum (Sigma) and 1% penicillin/streptomycin (Sigma) at 37°C in a humidified 5% CO_2 atmosphere. The medium was replaced at 48 h intervals, and the cells were always subcultured when the monolayer become confluent.

Leucine

The experimental leucine-rich mediums were composed by DMEM enriched with 25 or 50 μM leucine (Sigma), which were tested by ninhydrin method (Rosen 1957) to check the concentration of the tests solutions.

Cytotoxicity assay

The cell viability of control and leucine-treated Vero cells was assessed using the crystal violet method described by Murakami et al. (1998). Vero cell suspensions containing 1.5×10^5 cells/mL in 100 μL of DMEM medium supplemented with 5% fetal bovine serum and 1% penicillin/streptomycin were transferred to a 96-well culture plate (Corning) and cultured for 24, 48 and 72 h (control cells). Treated-cells were incubated with 25 or 50 μM leucine-rich medium, designed L25 and L50, respectively, and were grown under the same conditions as control cells. After incubation, the culture medium was removed and the cells were fixed in 10% formalin (Labsynth), stained with 0.05% crystal violet (Riedel-de Haën) in 20% methanol (Merck). The cells were then washed in 0.1 M PBS (phosphate buffered saline), pH 7.4, incubated in 0.1 M sodium citrate, pH 4.2, in 50% ethanol (Labsynth) for 30 min and the absorbance was measured at 540 nm in a microplate reader (Luminometer Fluorimeter Photometer Fusion^{TM}, Universal Microplate Analyser, Packard Bioscience Company, Meriden, USA).

Phase contrast and scanning electron microscopy

Vero cells were seeded at a density of 1.5×10^5 cells in 25-cm^2 tissue culture flasks (Corning) and treated with 25 or 50 μM leucine-rich medium for 24, 48 and 72 h. The morphological characteristics were assessed by observation of the cultures using an inverted phase contrast microscope (Leica Microsystems, Wetzlar, Germany).

Vero cells were seeded in a 24-well culture plate (Corning) provided with coverslips, at a density of 75×10^5 cells/mL and treated with 25 or 50 μM leucine-rich medium for 24, 48 and 72 h. After incubation, the cells were fixed in 2.5% glutaraldehyde (Sigma) in 0.1 M PBS, pH 7.4, washed in PBS and post-fixed in 1% osmium tetroxide (Sigma), dehydrated in a graded ethanol series (Labsynth), critical point dried (CDT 030, Balzers, BAL-TEC AG, Wiesbaden, Germany) and coated with gold in a sputter coater (CDT 050, Balzers). The cells were then analysed with a scanning electron microscope (JSM 5800 LV, JEOL, Peabody, USA).

Growth curves

Vero cells were seeded at a density of 25×10^3 cells/mL in 24-well culture plates (Corning) and treated with 25 or 50 μM leucine-rich medium for 24, 48 and 72 h. After incubation, the cells were cultured for 1–7 days. At selected times, the number of viable cells was counted in a hemocytometer chamber and the unviable cells were excluded based on the uptake of trypan blue. The growth curves obtained were the average of triplicate samples for each time interval.

Alkaline phosphatase activity

After 24, 48 and 72 h of incubation with 25 or 50 μM leucine-rich medium, Vero cells were homogenized in cold 0.1 M PBS, pH 7.4, and centrifuged at 10,000 rpm for 15 min at 4°C. The resulting supernatant was analysed for total protein content (Lowry et al. 1951). The alkaline phosphatase activity was measured using 30 μL of the homogenate supernatant with 37 mM *p*-nitrophenylphosphate (pNPP, Sigma) as substrate, and results being

expressed as percentage of the control cells (Martins et al. 2001).

Activity of proteolysis pathways

Measurement of proteasome activity

Vero cells, processed as described for measurement of alkaline phosphatase activity, were analysed as the functional proteasome activity, measuring the chymotrypsin-like enzyme activity, the predominant proteolytic activity of the β subunits of the proteasome, according to the method of Orino et al. (1991). Aliquots of the homogenate supernatant (50 μL) were used to determine chymotrypsin-like activity by incubating with 100 μL of the fluorogenic substrate succinyl-Leu-Leu-Val-Try-7-amino-4-methyl-coumarin (Suc LLVY-AMC, Sigma, 0.167 μg/L in Tris–HCl, Promega, pH 7.4) followed by the release of amino-methyl coumarin (AMC) from the substrate. The fluorescence was measured in a fluorimeter (excitation: 360 nm, emission: 460 nm) with the results being expressed as percentage of the control cells.

Measurement of lysosomal and calcium-dependent pathways activities

The lysosomal and calcium-dependent enzymes activities were measured in Vero cells, processed as described for measurement of alkaline phosphatase activity. The cathepsin B activity was determined using 50 μL of the homogenate supernatant treated with assay buffer (352 mM KH_2PO_4, 48 mM Na_2HPO_4, 4 mM EDTA pH 6.0 and 8 mM cistein) and prewarmed at 40°C for 2 min. The assay was started with the addiction of Z-Phe-Arg-NMec (Z-Phe-Arg 7-amido-4-methylcoumarin hydrochloride, Sigma) as substrate, dissolved in dimethyl sulphoxide and diluted to 0.02 mM with 0.1% Brij 35. After 10 min incubation at 40°C the fluorescence was measured in a fluorimeter (excitation: 360 nm, emission: 460 nm) at each min during 30 min (Barrett 1980). The only changes made for cathepsin H were the substitution of the substrate Arg-NMec (L-Arginine-7-amido-4-methyl-coumarin hydrochloride, Sigma), and the composition of the assay buffer (200 mM KH_2PO_4 and 200 mM Na_2HPO_4 adjusted at pH to 6.8) (Barrett 1980). The cathepsins B and H activities were expressed as percentages of the control cells. The calpain activity was measured using 50 μL of the homogenate supernatant in 50 mM imidazole–HCl buffer (pH 7.5), containing 10 mM b-mercaptoethanol, 1.0 mM NaN_3 and 4 mg/mL of casein. After incubation, a final concentration of 5 mM of $CaCl_2$ was then added to activate calpain and initiate the calpain hydrolysis reaction. Time scanning at a wavelength of 500 nm was performed at each min during 30 min (Jiang et al. 1997). The calpain activity was expressed as percentage of the control cells.

Measurement of total protein synthesis

Vero cells used for protein synthesis were grown to confluence in 24-well plates then exposed to leucine-rich medium (25 or 50 μM) and cultured for 24 h. After this period, the cells were analysed for protein synthesis using procedures adapted from White et al. (1988) with the following modifications: Vero cells were labelled with L-[2,6^{3}H] phenylalanine (0.67 μCi/mM in DMEM added with 2 mM cold phenylalanine) for 1 h. Subsequently, the cells were washed twice with 10% trichloroacetic acid, and then were removed from the plates and centrifuged. The cellular pellet was prepared by protein precipitation with 10% trichloroacetic acid, which was removed and 0.5 M NaOH was added together with 0.1% Triton and the incubation was carried out for 2 h at 37°C. The protein bound radioactivity was determined in 3 mL scintillation fluid, and expressed as percentage of the control cells.

Measurement of total protein degradation

The Vero cells were analysed for protein degradation using procedures from Gomes-Marcondes et al. (2002). The cells grown in 24-well plates were labelled with L-[2,6^{3}H] phenylalanine (0.67 μCi/mM in DMEM added with 2 mM cold phenylalanine) in 1 mL DMEM containing 5% fetal bovine serum. After 19 h, the cells were washed in PBS followed by the addition of fresh DMEM without phenol red, and then the cells were subsequently exposed to leucine-rich medium (25 or 50 μM) for 24 h. The protein degradation was determined measuring the radioactivity of the cell supernatant released into the medium using a Beckman LS 6000TA liquid scintillation counter, and expressed as percentage of the control cells.

Statistical analysis

The results were expressed as the mean ± SEM. One-way ANOVA followed by Bonferroni's test was used to compare the leucine-treated Vero cells with the controls (Gad and Weil 1994). A value of $P < 0.05$ indicated significance.

Results

Cytotoxicity assay

Leucine, at the concentrations used in the assays, has no cytotoxic effect on Vero cells over the time periods

analysed. No differences on cell viability were observed in L25 or L50 Vero cells compared to control cells after 24, 48 and 72 h of incubation (Fig. 1).

Phase contrast and scanning electron microscopy

The control Vero cells grew in a monolayer until confluence and showed the typical elongated shape of fibroblastic cells. After exposure to leucine-rich medium, the L25 and L50 Vero cells showed no alterations in their morphological characteristics after 24, 48 and 72 h incubation (light microscopy analysis not shown). The scanning microscopy analyses showed that the control and leucine-treated Vero cells formed similar ultrastructural characteristics of monolayers independent of the treatement, showing a regular-shaped, flattened cells containing vesicles and microvilli on their surface (Fig. 2: control cells A, D and G images; L25 cells B, E and H images; L50 cells C, F and I images).

Growth curves and alkaline phosphatase activity

The growth curves of control and leucine-treated cells are shown in the Fig. 3. The growth was similar at the beginning of the curves (first day after incubation; day 1); however, L25 and L50 Vero cells showed higher growth, especially after 48 and 72 h incubation (Fig. 3b, c), between days 2 and 6. The proliferation peaks were followed by senescence and cell death (days 6–7). In addition, as presented in Fig. 4a, inferring the cellular activity, there was a significant increase in leucine-treated Vero cells on alkaline phosphatase activity after 24 h of incubation with 25 μM leucine-rich medium, and after 48 and 72 h of incubation with 25 and 50 μM leucine-rich medium.

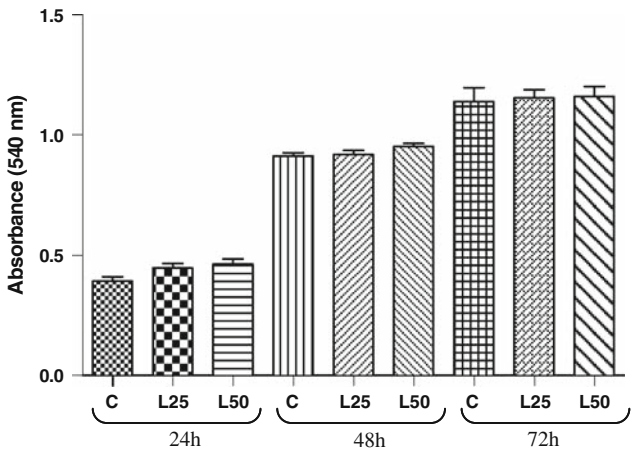

Fig. 1 Cytotoxicity assay in Vero cells after treatment with leucine for 24, 48 and 72 h. C: control; L25: Vero cell treated at 25 μM leucine-rich medium; L50: Vero cell treated at 50 μM leucine-rich medium. For details, see "Materials and methods". The *columns* represent the means ± SEM

Total protein synthesis and degradation

The Vero cells protein synthesis and degradation were compared between the control and the leucine-treated cells, after 24 h incubation. The rate of incorporation of [^{3}H]-phenylalanine by the Vero cells, indicative of protein synthesis, was enhanced in all leucine-treated cells, around 59 and 33% higher at 25 and 50 μM leucine-rich medium incubation, respectively (Fig. 4b). In contrast, representing the protein degradation, the release of [^{3}H]-Phe from the cell cultures was analysed in Vero cells and showed lower rate in both leucine-treatement when compared to the control (reduction around 80 and 63% in L25 and L50 cells after 24 h incubation, respectively) (Fig. 4b).

Activity of proteolysis pathways

Proteasome activity

The chymotrypsin-like activity, which corresponds to the catalytic core of 20S subunit of ubiquitin–proteasome pathway, was significantly decreased in L25 and L50 Vero cells, after 24, 48 and 72 h incubation (Fig. 5a) when compared to the control cells.

Lysosomal and calcium-dependent pathways activities

The cathepsins B and H enzymes activity correspond to the activity of the lysosomal protein degradation pathway in normal situation or by some stimuli, in this case by different leucine-rich medium concentration. The cathepsin B activity was significantly decreased in L 25 and L50 Vero cells 24, 48 and 72 h (Fig. 5b), while the cathepsin H activity (Fig. 5c) was decreased at both leucine-rich medium incubation only after 72 h, when compared to the control Vero cells. The calpain activities, which correspond to a group of cytosolic calcium-dependent proteases (Fig. 5d), were reduced in L25 and L50 Vero cells only after 72 h of incubation.

Discussion

The present findings clearly indicate that leucine stimulates the Vero cells proliferation and activity, and additionally modulates the ubiquitin–proteasome pathway, as well as the lysosomal pathway and proteolysis calcium-dependent system associated to an important reduction on protein degradation. These results clearly enforce the new approach

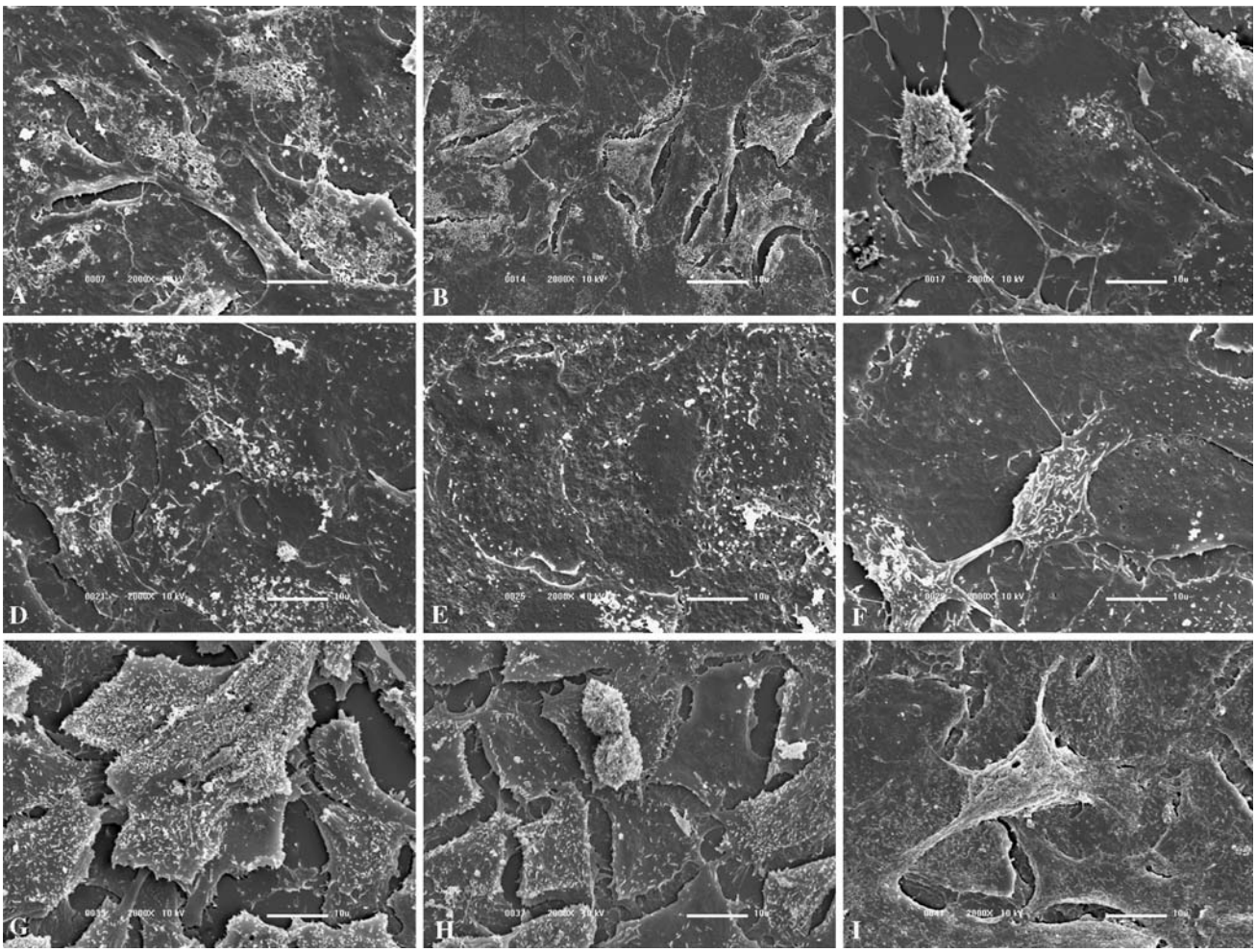

Fig. 2 Scanning electron microscopy: scanning electron micrographs of control and leucine-treated Vero cells. **a**, **d** and **g** control Vero cells after 24, 48 and 72 h of incubation, respectively. **b**, **e** and **h** Vero cells treated with 25 μM leucine-rich medium for 24, 48 and 72 h, respectively. **c**, **f** and **i** Vero cells incubated with 50 μM of leucine-rich medium for 24, 48 and 72 h, respectively. ×2,000. *Horizontal bars* = 10 μm

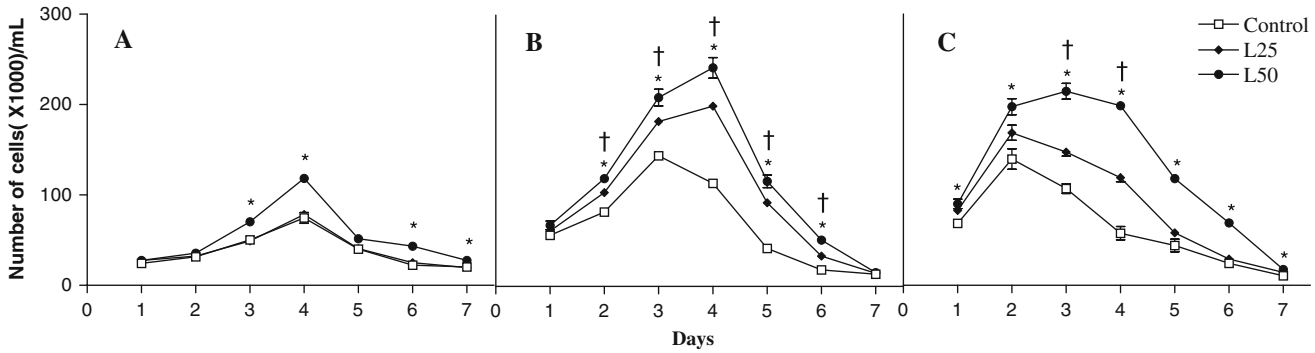

Fig. 3 Growth curves of control and leucine-treated Vero cells for 24, 48 and 72 h (**a**–**c**, respectively). The treated cells showed a higher growth rate than control cells especially from days 2 to 6. C: control; L25: Vero cell treated at 25 μM leucine-rich medium; L50: Vero cell treated at 50 μM leucine-rich medium. For details, see "Materials and methods". The *points* represent the means ± SEM. The statistical difference was expressed as (*dragon*) L25, (*asterisk*) L50 for $P < 0.05$ compared to control Vero cells

using fibroblastic cells to study other cells involved in wasting process as verified in cancer cachexia. Therefore, the effects of branched-chain amino acids on cellular metabolism and proliferation in vitro indicate a possible role of leucine on mitogenic signalling, as well as on protein metabolism in Vero cell.

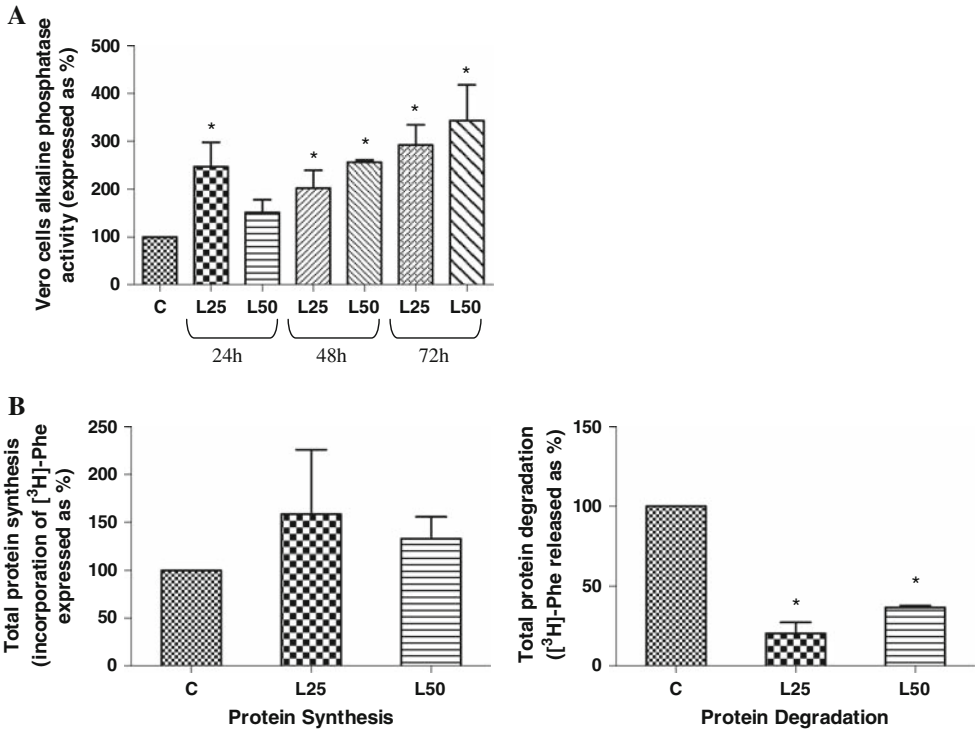

Fig. 4 Alkaline phosphatase activity and protein synthesis and degradation: **a** alkaline phosphatase activity in Vero cells, control or leucine-treated cultures for 24, 48 and 72 h. The control cells are set as 100% of alkaline phosphatase activity for each period analysed. **b** Total protein synthesis and proteolysis rate in Vero cell treated or not with 25 or 50 μM leucine-rich medium after 24 h incubation. The results are expressed as percentage of the control cells. C: control; L25: Vero cell treated at 25 μM leucine-rich medium; L50: Vero cell treated at 50 μM leucine-rich medium. For details, see "Materials and methods". The *columns* represent the means ± SEM. * $P < 0.05$ compared to control Vero cells

Xu et al. (1998) proposed that branched-chain amino acids may promote β-cell proliferation either by stimulating phosphorylation of PHAS-I (phosphorylated heat- and acid-stable protein regulated by insulin) and $p70^{s6k}$ (phosphorylated 70-kDa ribosomal protein S6 protein kinase) via the mammalian target of rapamycin (mTOR) pathway and/or by facilitating the proliferative effect mediated by growth factors such as insulin and IGF-I (insulin-like growth factor-I). The authors demonstrated that both insulin-secreting β-cells and insulin target cells (skeletal muscle, adipocytes, and liver) utilized similar, maybe identical, signalling pathways proposing the up-regulate protein translation, cell cycle progression, and cell proliferation.

Amino acids have been shown to activate components of the phosphatidylinositol (PI) 3-kinase-mammalian target of rapamycin (mTOR) signalling pathway, leading to enhanced muscle protein synthesis (Cantley 2002). Although it is clear that amino acids, in particular leucine, enhance phosphorylation of proteins downstream of mTOR, e.g., 4E-BP1 (eIF4E-binding protein 1) and S6K1 (70-kDa ribosomal protein S6 kinase), and indeed require mTOR to be active in order to be effective, whether or not they directly regulate mTOR activity is unclear (Bolster et al. 2004).

In addition, Kimura and Ogihara (2005) observed that leucine induces hepatocyte DNA synthesis and proliferation, and stimulates TGF-alpha (transforming growth factor-alpha) secretion. After utilization of cell-signalling specific inhibitors, the authors have evidenced that phospholipase C, tyrosine kinase, phosphatidylinositol 3-kinase, mitogen-activated protein (MAP) kinase, and $p70^{s6k}$ were involved in leucine signalling. The authors suggested that leucine triggers TGF-alpha secretion through a putative leucine receptor. The secreted TGF-alpha then stimulates hepatocyte DNA synthesis and proliferation through activation of TGF-alpha receptor to induce tyrosine kinase/MAP kinase activity and other downstream growth-related signal transducers.

Taken together, these findings in literature may be related to the ability of leucine to increase proliferation in Vero cell cultures, as observed in our study. Focusing the increased on alkaline phosphatase activity, which can indicate the cellular activity, such as cellular transport and absorption (Calhau et al. 2002; Toledo and Gomes-Marcondes 2004), and the growth curves, especially after 48 and 72 h leucine-rich medium exposition, the Vero cells clearly showed the enhance on the cell proliferation; therefore, the leucine-signalling pathway inducing Vero cells proliferation remains to be elucidated.

Especially related to the cell activity, including proliferation and synthesis, as also verified in the present work, the leucine can interfere in many cellular process, such as protein turnover and cell cycle, maybe reducing the cell death, as this amino acid and also its metabolite β-hydroxy-β-methylbutyrate can inhibit the protein cell degradation in vitro and in vivo, as well stimulating the protein synthesis (Kanazawa et al. 2004; Smith et al. 2004, 2005; Ventrucci et al. 2004, 2007). Knowing that the intracellular protein

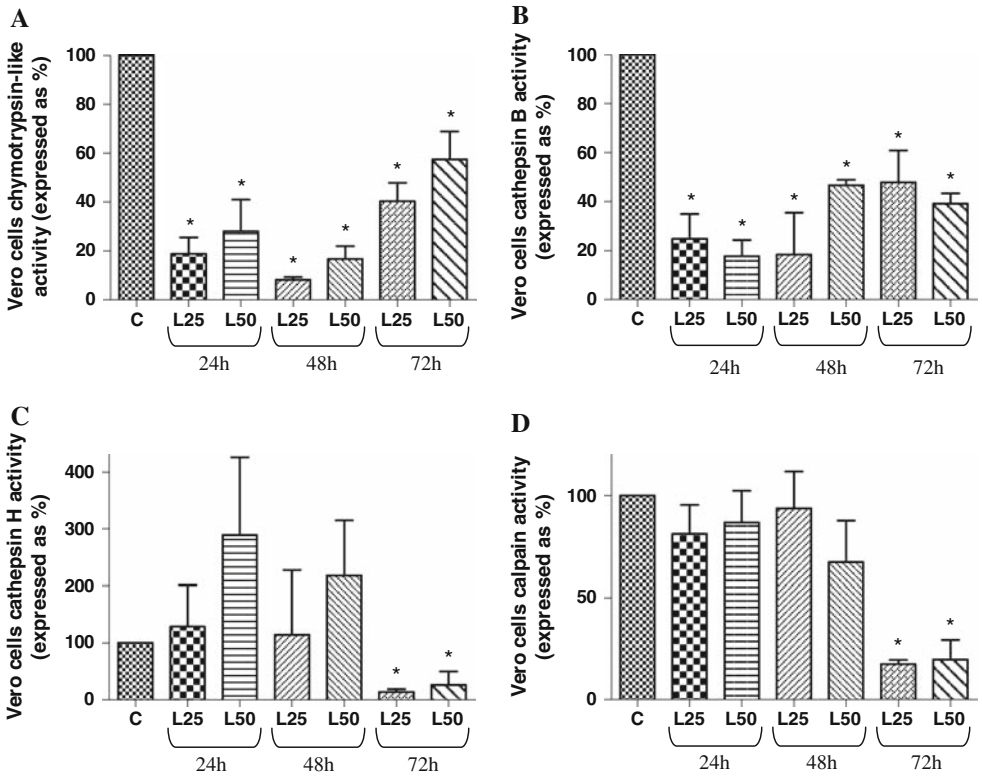

Fig. 5 Analysis of proteolysis systems in Vero cells culture: effect of leucine on proteasome functional activity, determined as the chymotrypsin-like enzyme activity in Vero cells treated with 25 or 50 μM leucine-rich medium for 24, 48 and 72 h and compared to control Vero cells (100% of chymotrypsin-like activity for each period analysed). Vero cells chymotrypsin-like activity was significantly decreased in L25 and L50 Vero cells at 24, 48 and 72 h incubation. **b** Vero cells cathepsin B, cathepsin H (**c**), and calpain activities (**d**) were significantly decreased in L25 and L50 Vero cells for 72 h (control cells are set as 100% of enzyme activity for each period analysed). C: control; L25: Vero cell treated at 25 μM leucine-rich medium; L50: Vero cell treated at 50 μM leucine-rich medium. For details, see "Materials and methods". The *columns* represent the means ± SEM. * $P < 0.05$ compared to control Vero cells

degradation depends on three major proteolytic systems: the lysosomal pathway, the calcium-dependent pathway, and the ATP-ubiquitin–proteasome-dependent pathway (Hasselgren and Fischer 2001), the leucine exposure could modulate these processes improving the cell ability to generate more cell activity such the cell cycle. The present work investigated the protein degradation in fibroblastic cells analysing the proteolysis ATP-dependent ubiquitin pathway, which involves an enzymatic cascade by which multiple ubiquitin molecules are covalently attached to the protein target, which is then degraded by the 26S proteasome complex. The catalytic core of the 26S proteasome is the 20S proteasome, characterized by five peptidase activities: the trypsin-like, chymotrypsin-like, peptidyl-glutamyl peptidase, branched-chain amino acid-preferring, and small neutral amino acid-preferring activities (Jagoe and Goldberg 2001). The ubiquitin–proteasome system is considered to be the most important pathway for intracellular protein degradation in catabolic conditions (Lecker et al. 1999). Studies showed that leucine could reduce the proteolysis in chick myotubes (Nakashima et al. 2005) and in muscle tissue (Ventrucci et al. 2004) by inhibiting the ubiquitin and proteasome subunits expression (Nakashima et al. 2005). Equally, the leucine metabolite, β-hydroxy-β-methylbutyrate, also attenuated the proteasome-induced proteolysis by PIF in myotubes (Smith et al. 2004) and also in muscle of tumour-bearing mice (Smith et al. 2005). Both amino acid or it's metabolite induce the modulation of ubiquitin–proteasome pathway inhibiting the chymotrypsin-like activity as well as the 19S and 11S proteasome subunits in vitro and in vivo (Nakashima et al. 2005; Smith et al. 2004, 2005; Ventrucci et al. 2004). Conversely, the fluorogenic substrate specific for a proteasome proteolytic activity (chymotrypsin-like activity) is less cleaved in L25 and L50 Vero cells than in control cells, demonstrating that functional proteasome activity is significantly modulated in the presence of leucine, indicating that even in normal cell condition the leucine can improve the cell activities managing this proteolytic system or other mechanisms to cell cycle or proliferation more efficiently.

The other two systems, which are also involved to the protein degradation, are the lysosomal system and calcium-dependent pathway. The lysosomal is concerned to proteolysis of extracellular proteins and cell surface receptors

and is not involved in the breakdown of myofibrillar proteins in muscle cells (Bechet et al. 2005; Lowell et al. 1986); parallelely the lysosomal cystein cathepsins are also involved in signalling pathway in apoptosis (Chwieralski et al. 2006; Stoka et al. 2007). Under physiological conditions these enzymes are synthesized as pre-pro-enzymes and are localized intralysosomally being released to cytosol by certain stimulus (Chwieralski et al. 2006). The analysis of lysosomal pathway showed that leucine could modulate this process in Vero cells. These facts are also verified by other authors that amino acids suppressed the autophagic proteolysis in isolated hepatocytes (Kadowaki and Kanazawa 2003; Kanazawa et al. 2004) or during leucine depletion the autophagic process was enhanced in C_2C_{12} myotubes (Mordier et al. 2000). Amino acids, especially leucine, in addition to their ability to function as substrates for protein synthesis, are also inhibitors of autophagic protein degradation (Kadowaki and Kanazawa 2003; Kim et al. 2008; Meijer and Codogno 2008). As extensively reviewed by Meijer and Codogno (2004, 2006, 2008), amino acids (leucine in particular), in combination with insulin or other growth factors, can stimulate mTOR signalling, although the inhibition of mTOR activity by rapamycin (or amino acid depletion) stimulates autophagy. Similarly, since the lysosomal cathepsin B activities were decreased in Vero cells after leucine-rich medium treatment, the apoptosis process, which could be cathepsin signalled (Chwieralski et al. 2006; Stoka et al. 2007), is probably depressed in these cells confirming the cell progression increase (Fig. 3) associated to the increase in cell activity (enhanced alkaline phosphatase activity inferring the cell activity; Fig. 4a). Calcium-dependent proteases have been proposed to be required in the initial degradation of skeletal myofibrillar proteins during sepsis (Williams et al. 1999) and have been found to be activated in the skeletal muscle and heart of tumour-bearing rats (Costelli et al. 2001), both catabolic states; our results displayed significantly decrease on calpain activities in Vero cells treated with leucine-rich culture medium. Nakashima et al. (2005) also demonstrated that leucine inhibited the calpain subunit, but not cathepsin mRNA expression in cultured chick myotubes. The present results indicate that leucine is capable to attenuate the global Vero cells proteolysis and probably the cell death, suggesting that these proteolysis pathways could be modulated by leucine in correspondence to the improvement of other cellular process such as cell cycle rather than apoptosis. Our findings clearly emphasize the importance of leucine alone to mediate protein synthesis and restrain catabolism in Vero cells cultures.

In conclusion, our results demonstrated that the lysosomal, the calcium-dependent, the ATP-dependent ubiquitin–proteasome proteolytic pathways and the total protein degradation were modulated by leucine in Vero cells and this modulation was associated to an increase in cell proliferation, total protein synthesis and alkaline phosphatase activity. These data strengthen the view that leucine may be useful for anticatabolic therapy in catabolic conditions such as sepsis, severe injury and cancer cachexia. Our observations also support the concept that Vero cells may represent a new model of the proteolysis pathways study, especially focusing the extracellular matrix, which also can be wasted in cancer cachexia. However, the precise mechanism by which leucine can induce cell proliferation and modulate the protein breakdown by proteolytic systems in Vero cells needs to be better understood. The investigations are currently underway in our laboratory to address these important questions.

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References

Anthony TG, Anthony JC, Yoshizawa F, Kimball SR, Jefferson LS (2001) Oral administration of leucine stimulates ribosomal protein mRNA translation but not global rates of protein synthesis in the liver of rats. J Nutr 131:1171–1176

Avruch J, Long X, Ortiz-Vega S, Rapley J, Papageorgiou A, Daí N (2008) Amino acid regulation of TOR complex 1. Am J Physiol Endocrinol Metab. doi:10.1152/ajpendo.90645.2008

Barrett AJ (1980) Fluorimetric assays for cathepsin B and cathepsin H with methylcoumarylamide substrates. Biochem J 187:909–912

Bechet D, Tassa A, Taillandier D, Combaret L, Attaix D (2005) Lysosomal proteolysis in skeletal muscle. Int J Biochem Cell Biol 37:2098–2114

Biolo G, De Cicco M, Dal Mas V, Lorenzon S, Antonione R, Ciocchi B, Barazzoni R, Zanetti M, Dore F, Guarnieri G (2006) Response of muscle protein and glutamine kinetics to branched-chain-enriched amino acids in intensive care patients after radical cancer surgery. Nutrition 22:475–482

Bolster DR, Jefferson LS, Kimball SR (2004) Regulation of protein synthesis associated with skeletal muscle hypertrophy by insulin-, amino acid- and exercise-induced signalling. Proc Nutr Soc 63:351–356

Calhau C, Martel F, Hipólito-Reis C, Azevedo I (2002) Modulation of uptake of organic cationic drugs in cultured human colon adenocarcinoma Caco-2 cells by an ecto-alkaline phosphatase activity. J Cell Biochem 87:408–416

Cantley LC (2002) The phosphoinositide 3-kinase pathway. Science 296:1655–1657

Chwieralski CE, Welte T, Bühling F (2006) Cathepsin-regulated apoptosis. Apoptosis 11:143–149

Costelli P, Tullio RD, Baccino FM, Melloni E (2001) Activation of $Ca^{(2+)}$-dependent proteolysis in skeletal muscle and heart in cancer cachexia. Br J Cancer 84:946–950

Gad SC, Weil CS (1994) Statistics for toxicologists. In: Wallace H (ed) Principles and methods of toxicology. Raven Press, New York

Gomes-Marcondes MC, Smith HJ, Cooper JC, Tisdale MJ (2002) Development of an in-vitro model system to investigate the mechanism of muscle protein catabolism induced by proteolysis-inducing factor. Br J Cancer 86:1628–1633

Gomes-Marcondes MC, Ventrucci G, Toledo MT, Cury L, Cooper JC (2003) A leucine-supplemented diet improved protein content of skeletal muscle in young tumour-bearing rats. Braz J Med Biol Res 36:1589–1594

Harper AE, Miller RH, Block KP (1984) Branched-chain amino acid metabolism. Ann Rev Nutr 4:409–454

Hasselgren PO, Fischer JE (2001) Muscle cachexia: current concepts of intracellular mechanisms and molecular regulation. Ann Surg 233:9–17

Ichihara A (1999) BCA, HGF, and proteasomes. Biochem Biophys Res Commun 266:647–651

Jagoe RT, Goldberg AL (2001) What do we really know about the ubiquitin proteasome pathway in muscle atrophy. Curr Opin Clin Nutr Metab Care 4:183–190

Jiang ST, Wang JH, Chang T, Chen CS (1997) A continuous method for measuring calpain activity. Anal Biochem 244:233–238

Kadowaki M, Kanazawa T (2003) Amino acids as regulators of proteolysis. J Nutr 133:2052S–2056S

Kanazawa T, Taneike I, Akaishi R, Yoshizawa F, Furuya N, Fujimura S, Kadowaki M (2004) Amino acids and insulin control autophagic proteolysis through different signaling pathways in relation to mTOR in isolated rat hepatocytes. J Biol Chem 279:8452–8459

Kim E, Goraksha-Hicks P, Li L, Neufeld TP, Guan KL (2008) Regulation of TORC1 by Rag GTPases in nutrient response. Nature Cell Biol 10:935–955

Kimball SR, Jefferson LS (2006) New functions for amino acids: effects on gene transcription and translation. Am J Clin Nutr 83:500S–507S

Kimura M, Ogihara M (2005) Effects of branched-chain amino acids on DNA synthesis and proliferation in primary cultures of adult rat hepatocytes. Eur J Pharmacol 510:167–180

Kobayashi H, Kato H, Hirabayashi Y, Murakami H, Suzuki H (2006) Modulations of muscle protein metabolism by branched-chain amino acids in normal and muscle-atrophying rats. J Nutr 136:234S–236S

Layman DK, Baum JI (2004) Dietary protein impact on glycemic control during weight loss. J Nutr 134:968S–973S

Lecker SH, Solomon V, Mitch WE, Goldberg AL (1999) Muscle protein breakdown and the critical role of the ubiquitin-proteasome pathway in normal and disease states. J Nutr 129:227–237

Lowell BB, Ruderman NB, Goodman MN (1986) Evidence that lysosomes are not involved in the degradation of myofibrillar proteins in rat skeletal muscle. Biochem J 234:237–240

Lowry O, Rosebrough NJ, Farr AL, Randall RL (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275

Martins MJ, Negrão MR, Hipólito-Reis C, Azevedo I (2001) Arginine and a polyarginine peptide inhibit alkaline phosphatase activity: possible consequences for cellular transport systems. Clin Biochem 34:435–437

May ME, Buse MG (1989) Effects of branched-chain amino acids on protein turnover. Diabetes Metab Rev 5:227–245

Meijer AJ, Codogno P (2004) Regulation and role of autophagy in mammalian cells. Int J Biochem Cell Biol 36:2445–2462

Meijer AJ, Codogno P (2006) Signalling and autophagy regulation in health, aging and disease. Mol Aspects Med 27:411–425

Meijer AJ, Codogno P (2008) Nutrient sensing: TOR's ragtime. Nat Cell Biol 10:881–883

Mordier S, Deval C, Bechet D, Tassa A, Ferrara M (2000) Leucine limitation induces autophagy and activation of lysosome-dependent proteolysis in C2C12 myotubes through a mammalian target of rapamycin-independent signaling pathway. J Biol Chem 275:29900–29906

Murakami N, Fukuchi S, Takeuchi K, Hori T, Shibamoto S, Ito F (1998) Antagonistic regulation of cell migration by epidermal growth factor and glucocorticoid in human gastric carcinoma cells. J Cell Physiol 176:127–136

Nakai N, Shimomura Y, Tamura T, Tamura N, Hamada K, Kawano F, Ohira Y (2006) Leucine-induced activation of translational initiation is partly regulated by the branched-chain alpha-keto acid dehydrogenase complex in C2C12 cells. Biochem Biophys Res Commun 343:1244–1250

Nakashima K, Ishida A, Yamazaki M, Abe H (2005) Leucine suppresses myofibrillar proteolysis by down-regulating ubiquitin-proteasome pathway in chick skeletal muscles. Biochem Biophys Res Commun 336:660–666

Orino E, Tanaka K, Tamura T, Sone S, Ogura T, Ichihara A (1991) ATP-dependent reversible association of proteasomes with multiple protein components to form 26S complexes that degrade ubiquitinated proteins in human HL-60 cells. FEBS Lett 284:206–210

Proud CG (2007) Amino acids and mTOR signalling in anabolic function. Biochem Soc Trans 35:1187–1190

Rosen H (1957) A modified ninhydrin colorimetric analysis for amino acids. Arch Biochem Biophys 67:10–15

Smith HJ, Wyke SM, Tisdale MJ (2004) Mechanism of the attenuation of proteolysis-inducing factor stimulated protein degradation in muscle by β-hydroxy-β-methylbutyrate. Cancer Res 64:8731–8735

Smith HJ, Mukerji P, Tisdale MJ (2005) Attenuation of proteasome-induced proteolysis in skeletal muscle by β-hydroxy-β-methylbutyrate in cancer-induced muscle loss. Cancer Res 65:277–283

Stipanuk MH (2007) Leucine and protein synthesis: mTOR and beyond. Nutr Rev 65:122–129

Stoka V, Turk V, Turk B (2007) Lysosomal cysteine cathepsins: signalling pathways in apoptosis. Biol Chem 388:555–560

Sun X, Zemel MB (2007) Leucine and calcium regulate fat metabolism and energy partitioning in murine adipocytes and muscle cells. Lipids 42:297–305

Toledo MT, Gomes-Marcondes MCC (2004) Placental glycogen metabolism changes during Walker tumour growth. Placenta 25:456–462

Ventrucci G, Mello MA, Gomes-Marcondes MC (2001) Effects of leucine-supplemented diet on body composition changes in pregnant rats bearing Walker 256 tumor. Braz J Med Biol Res 34:333–338

Ventrucci G, Mello MA, Gomes-Marcondes MC (2004) Proteasome activity is altered in skeletal muscle tissue of tumour-bearing rats fed a leucine-rich diet. Endocr Relat Cancer 11:887–895

Ventrucci G, Mello MA, Gomes-Marcondes MC (2007) Leucine-rich diet alters the eukaryotic translation initiation factors expression in skeletal muscle of tumour-bearing rats. BMC Cancer 7:42

White ME, Allen CE, Dayton WR (1988) Effect of sera from fed and fasted pigs on proliferation and protein turnover in cultured myogenic cells. J Anim Sci 66:34–42

Williams AB, Decourten-Myers GM, Fischer JE, Luo G, Sun X, Hasselgren PO (1999) Sepsis stimulates release of myofilaments in skeletal muscle by a calcium-dependent mechanism. FASEB J 13:1435–1443

Xu G, Kwon G, Marshall CA, Lin TA, Lawrence JC Jr, McDaniel ML (1998) Branched-chain amino acids are essential in the regulation of PHAS-I and p70 S6 kinase by pancreatic beta-cells. A possible role in protein translation and mitogenic signalling. J Biol Chem 273:28178–28184