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Leucine Limitation Induces Autophagy and Activation of Lysosome-dependent Proteolysis in C2C12 Myotubes through a Mammalian Target of Rapamycin-independent Signaling Pathway*

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Loss of muscle mass usually characterizes different pathologies (sepsis, cancer, trauma) and also occurs during normal aging. One reason for muscle wasting relates to a decrease in food intake. This study addressed the role of leucine as a regulator of protein breakdown in mouse C2C12 myotubes and aimed to determine which cellular responses regulate the process. Determination of the rate of protein breakdown indicated that leucine is one key regulator of this process in myotubes because starvation for this amino acid is responsible for 30–40% of the total increase generated by total amino acid starvation. Leucine restriction rapidly accelerates the rate of protein breakdown (+11 to 15% ($p < 0.001$) after 1 h of starvation) in a dose-dependent manner. By using various inhibitors, evidence is provided that acceleration of protein catabolism results mainly from an induction of autophagy, activation of lysosome-dependent proteolysis, without modification of mRNA levels encoding the lysosomal cathepsins B, L, or D. Those results suggest that autophagy is an essential cellular response for increasing protein breakdown in muscle following food deprivation. Induction of autophagy precedes a decrease in global protein synthesis (−20% to −30% ($p < 0.001$)) that occurs after 3 h of leucine starvation. Inhibition of the mammalian target of rapamycin (mTOR) activity does not abolish the effect of leucine starvation and the level of phosphorylated ribosomal S6 protein is not affected by leucine withdrawal. These latter data provide clear evidence that the mTOR signaling pathway is not involved in the mediation of leucine effects on both protein synthesis and degradation in C2C12 myotubes.

In higher eukaryotes, intracellular proteins undergo a continuous turnover, highly regulated by circulating hormones and nutrients. Protein degradation is performed by elaborate proteolytic systems located in multiple compartments. One characterized enzymatic system is the proteasome mainly localized in the cytosol. It is a multisubunit proteolytic complex composed of a central multicatalytic core, the 20 S proteasome, which can be associated to different regulatory complexes, especially the 19 S regulator (or PA700) to form the 26 S proteasome (1). One way for the targeting of substrates to the 26 S proteasome is their conjugation to ubiquitin (2).

Intracellular protein degradation also occurs in the lysosome, enriched in multiple hydrolytic enzymes, especially the cathepsins (3). The accession of substrates to the lysosome can be achieved by macroautophagy. During this process, cytosolic components are enclosed in structures generated from portions of the endoplasmic reticulum free of ribosomes, named autophagosomes, which then fuse with the lysosome (4). The process of autophagy started to be studied at the molecular level in yeast with the cloning of a series of genes, by complementation of autophagy-defective mutants (5). In particular, a new conjugation system involved in the formation of autophagosomes has been characterized (6). It is similar to the ubiquitin conjugation system, but involves a set of distinct, non-homologous proteins, named Apg(s) (7, 8). Those proteins are conserved in humans, and their genes are expressed in many tissues, including liver and skeletal muscle (9).

In the liver, autophagy is an important physiological response and some amino acids can regulate this sequestration pathway. Indeed, it has been shown that after amino acid withdrawal, liver perfusion with a group of eight amino acids leads to an inhibition of autophagy at the sequestration step (10, 11). Among them, leucine, phenylalanine, and tyrosine are the most potent to inhibit the formation of autophagosomes.

In skeletal muscle, amino acids, together with hormones, are key regulators of protein metabolism (12–14). The importance of branched chain amino acids, especially leucine, in the regulation of mRNA translation has been demonstrated in animal models (15, 16). Recent *in vitro* studies on rat L6 myoblasts revealed that leucine stimulates global protein synthesis through the regulation of the initiation factor eIF2B[1] (17). In addition, leucine activates the signal transduction pathway involving mTOR (18). The mTOR kinase regulates eIF4E availability via the phosphorylation of 4E-BP1 (19), as well as the translation of specific mRNAs through the activation of the $p70^{s6k}$ (20). The mTOR kinase also regulates yeast autophagy (21) and may control liver autophagy in response to amino acids (22).

Increased muscle protein breakdown associated with starvation generates free amino acids which enter the circulation. These amino acids supply peripheral tissues to maintain vital functions, from *de novo* protein synthesis to hepatic gluconeogenesis. Severe diseases, like cancer, trauma, or sepsis, are also characterized by a loss of skeletal muscle proteins that provide amino acids for wound healing and synthesis of acute phase proteins (23, 24). The involvement of the different proteolytic

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[1] The abbreviations used are: eIF, eukaryotic initiation factor; mTOR, mammalian target of rapamycin; 4E-BP1, eIF4E-binding protein; s6k, S6 kinase; S6-P, phosphorylated ribosomal S6; HS, horse serum; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline.

systems in muscle wasting has been extensively studied in various animal models (25). The ATP-proteasome-dependent pathway is activated in muscle of septic or cachectic animals (26–29). In models of fasting, this proteolytic pathway is activated as well, as a consequence of the induction of stress hormones like glucocorticoids that oppose the anabolic effects of insulin (30). Muscle lysosomal-dependent proteolysis is also elevated during fasting (30), and increased expression of mRNAs encoding some cathepsins has been observed in chronic septic phase (26) or in head trauma patients (31).

So far, few studies have addressed how the major intracellular proteolytic systems react to changes in the levels of specific amino acids independently of hormonal regulation. In view of the evidence that leucine regulates skeletal muscle protein synthesis, and the possible regulation of autophagy and protein synthesis through common signaling pathways, we addressed herein the following issues. Using the C2C12 mouse muscle cell line, we first established and characterized the contribution of leucine in the regulation of protein breakdown; second, we determined which cellular response(s), autophagy, and proteolytic system(s) are regulated by amino acids or leucine availability; third, we studied the involvement of the mTOR pathway in the adaptive response to leucine depletion. Evidence is given here for leucine as an essential regulator of protein breakdown in muscle, and the induction of autophagy as a cellular adaptation responsible for the increased lysosomal-dependent protein breakdown measured after amino acid starvation. Moreover, we demonstrate that leucine starvation regulates autophagy and protein synthesis through an mTOR-independent signaling pathway.

EXPERIMENTAL PROCEDURES

Cell Culture—The C2C12 mouse muscle cell line was from the ATCC. The C2C12 cells (30×10^3 cells/cm^2) were plated in 24-well dishes and grown for one day in DMEM containing 4.5 g/liter glucose, sodium pyruvate (1 mM), gentamycin (10 mg/liter), and 10% fetal calf serum. Fusion and differentiation of myoblasts into myotubes was then induced by replacing fetal calf serum in the medium with 5% horse serum (HS) for 2 days and then 2% HS for 2 more days. After 5 days, a fusion index of 70–80% was generally achieved.

Protein Degradation Measurement—At day 4 of differentiation, myotubes were incubated for 18 h (0.5 ml of medium/well) in differentiation medium containing 2% HS and 7 μCi of L-[^{35}S]Met/ml. Cells were rinsed once with culture medium. During the successive chase periods, cells were incubated with either DMEM/F12 (Sigma), medium lacking leucine prepared from DMEM/F12 base medium (Sigma), or medium lacking all amino acids (Life Technologies, Inc.). Each medium was supplemented with 2% dialyzed HS and 2 mM L-Met. Cells were first incubated for 1 h in complete medium as a control for identical K_d calculated as described below. A second chase was then performed during 3 h and eventually followed by additional ones performed with fresh medium for 10–21 h. The different culture media and chase periods used are indicated in the figure legends. Aliquots of culture medium (20 μl) were taken at different times during the successive chase periods. Proteins were precipitated at 4 °C with trichloroacetic acid (17% final) in the presence of 5 μg of bovine serum albumin. At the end of the chase, cells were rinsed once in PBS and also precipitated at 4 °C in 10% trichloroacetic acid. DNA and protein content as well as the residual radioactivity of cells were determined as described in Ref. 32. Protein degradation was represented in two different manners: 1) L-[^{35}S]Met release (percentage of total incorporated radioactivity) as a function of time (total radioactivity is the sum of the residual radioactivity of the cells and the trichloroacetic acid-soluble and insoluble radioactivities at different time points); and 2) K_d increase expressed as a percentage of variation relative to control during the time-course experiment. The rate of protein degradation, K_d, that represents the percentage of degraded protein per h, was determined from the slope of $\ln(A_0/A)$ as a function of time (formula $K_d \times t = \ln(A_0/A)$), where A_0 is the total incorporated L-[^{35}S]Met during the 18 h of labeling and A is the remaining L-[^{35}S]Met at time t. For cells cultured in complete medium, the percentage of protein degraded per hour (K_d) in all separate experiments equals 3.2 ± 0.6. Statistically significant differences were confirmed using Student's t test ($n = 6$). The following inhibitors were used for blocking the different proteolytic pathways. 3-Methyladenine (10 mM) inhibits autophagy, concanamycin A (1 μM) blocks the lysosomal proton pump and inhibits lysosome-dependent proteolysis, and lactacystin (2 μM) irreversibly inhibits proteasome enzymatic activities.

Protein Synthesis Measurement—Cells were incubated in various culture media for a given time as indicated in the figure legends. During the last 1 h, L-[^{35}S]Met was added (2 μCi/well for a 24-well plate). Cells were rinsed once in PBS and precipitated at 4 °C in 10% trichloroacetic acid. DNA and protein content, as well as incorporated L-[^{35}S]Met, were determined as described in Ref. 32. The rate of protein synthesis was calculated from the trichloroacetic acid-insoluble radioactivity incorporated per hour and per microgram of protein. Results were expressed as relative decreases in L-[^{35}S]Met incorporation compared with 100% of the controls.

Multiplex Messenger RNA Assay—Target DNAs were amplified by polymerase chain reaction using appropriate oligonucleotides, purified and denatured (95 °C for 10 min) before dotting on Hybond N+ membrane (50 ng for each cDNA). Dotted DNA was cross-linked by UV irradiation. Total RNA was extracted using guanidium thiocyanate (33). A synthetic mRNA of *Arabidopsis thaliana* cytochrome c_{554} (cDNA cloned in pHD-1 vector (kindly provided by H. Hofte, INRA Versailles, France)) was synthesized from the T3 promoter using the RiboMax large scale production system (Promega). Following oligo(dT) annealing, 15 μg of total RNA were reverse-transcribed for 2 h at 42 °C with Superscript II (Life Technologies, Inc.) in the presence of [α-^{32}P]dCTP. c554 mRNA was added to the reverse transcription reaction (0.25% of estimated mRNA) for normalization and quantification after hybridization. Following reverse transcription, total RNA was degraded by NaOH treatment and the complex probe purified by gel filtration on G50. Pre-hybridization (24 h) and hybridization (48 h) were performed at 68 °C in 5× SSC, 5× Denhardt's reagent, 0.5% SDS, sonicated DNA (100 μg/ml), and membranes were washed at 68 °C (one in 2× SSC, 0.1% SDS; one in 0.5× SSC, 0.1% SDS; one in 0.1× SSC, 0.1% SDS). Hybridization signals were detected using a PhosphorImager (Molecular Dynamics) and quantified using ImageQuant software.

Detection of Phosphorylated Proteins—C2C12 myoblasts were cultured on 60-mm plates and differentiated as described for 24-well plates. At day 5, an 8-h time course was performed using different culture media as indicated in the figure legends. Medium lacking all amino acids was supplemented with L-Met to reproduce the conditions used during catabolism measurement. Myotubes were scraped and disrupted in 150 μl of RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 50 mM NaF, 2 mM Na_3VO_4, 0.1 mM okadaic acid, 25 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 1 pg/ml leupeptin). Soluble proteins were recovered after a 10-min centrifugation (12,000 × g), and their concentration determined using BCA reagent (34). Equal amounts of protein were heated 10 min at 90 °C and analyzed in 12% SDS-polyacrylamide gel electrophoresis under reducing conditions (35), and then transferred to Hybond C. The membranes were blocked (0.2% nonfat dry milk in PBS), exposed to anti-S6P antiserum, washed and exposed to an alkaline phosphatase-conjugated anti-rabbit immunoglobulin. Fluorescent immunoconjugates were revealed using the ECF detection reagents (Amersham Pharmacia Biotech) and detected using a PhosphorImager and quantified using ImageQuant software.

RESULTS

Effects of Leucine Starvation on Protein Breakdown in C2C12 Myotubes: Comparison with Total Amino Acid Withdrawal—As leucine is a key regulator of protein synthesis in muscle, we aimed to determine whether it was also a regulator of protein breakdown in this tissue. Moreover, in order to estimate the relative contribution of leucine in the regulation of protein metabolism in muscle cells, the effect of its depletion was compared with that of total amino acids depletion. During a 24-h time-course experiment, the lack of amino acids or leucine in the culture medium induced a higher release of L-[^{35}S]Met as compared with control (Fig. 1*A*). The increase in protein breakdown for cells exposed to medium lacking all amino acids was between 20% and 35% ($p < 0.001$), compared with an 11–15% ($p < 0.001$) increase when cells were leucine-starved. In the same cells, the protein/DNA ratio was diminished by 9–15% after 24 h of leucine starvation compare with a 15–18% decrease for total amino acid starvation (Fig. 1*B*). These results demonstrate that leucine may be one key regu-

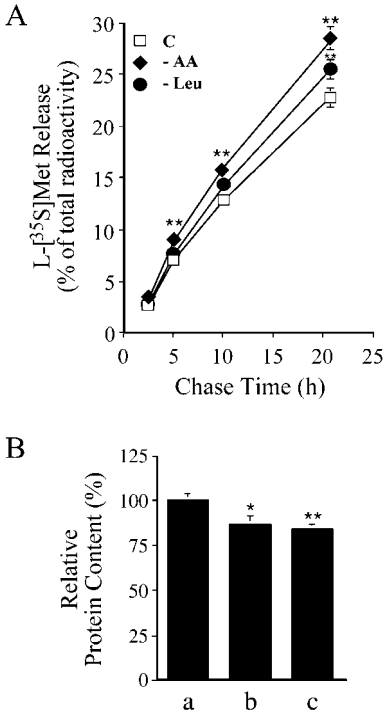


FIG. 1. **Effect of leucine or total amino acid starvation on protein breakdown in C2C12 myotubes.** *A*, L-[^{35}S]Met release from C2C12 myotubes as an index of total protein breakdown. Following protein labeling, myotubes were incubated in complete DMEM/F12 (□), medium lacking all amino acids (◆), or medium lacking leucine (●). Extracellular trichloroacetic acid-soluble radioactivity was measured during a 24-h time-course experiment and was expressed as a percentage of the total incorporated L-[^{35}S]Met. *B*, relative protein content after 24 h in complete DMEM/F12 (*a*), medium lacking all amino acids (*b*) or leucine only (*c*). All values are means ± SD ($n = 6$) and were reproduced in at least three independent experiments. * ($p < 0.005$) and ** ($p < 0.001$) indicate a statistically significant difference compared with the cells incubated in complete DMEM/F12.

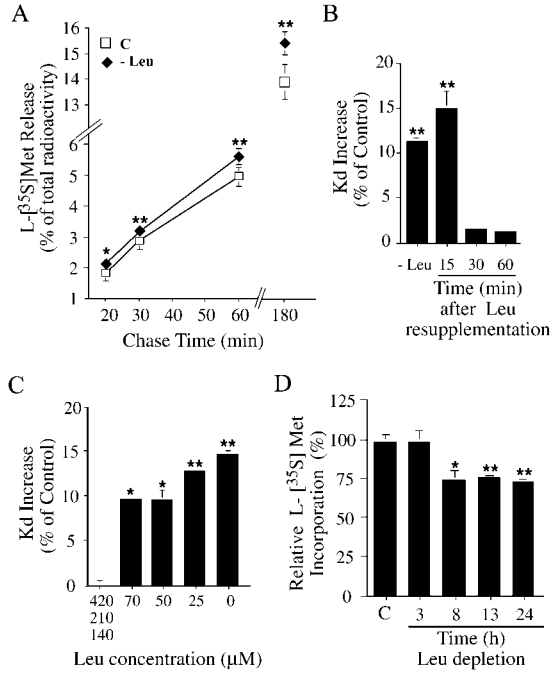


FIG. 2. **Characterization of the response to leucine availability in C2C12 myotubes.** *A*, L-[^{35}S]Met release from C2C12 myotubes as an index of total protein breakdown. Cells were incubated in either complete DMEM/F12 containing 420 μM leucine (□) or leucine-free medium (◆). Extra-cellular trichloroacetic acid-soluble radioactivity was measured during a 3-h time-course experiment as indicated and was expressed as a percentage of the total incorporated L-[^{35}S]Met. *B*, effect of leucine re-supplementation on protein breakdown. Cells were leucine-starved for 8 h and re-supplemented with leucine. K_d increase relative to cells in DMEM/F12 was determined at 8 h of leucine starvation (*−Leu*) and at the indicated times following supplementation. *C*, effect of leucine concentration on protein breakdown. After labeling, myotubes were incubated in medium containing the indicated leucine concentrations during a 13-h time-course experiment. The percentage of increase was deduced from the difference between the K_d in complete DMEM/F12 and the K_d at a given leucine concentration. *D*, relative protein synthesis was measured as described under "Experimental Procedures" and normalized to protein content, after 24 h in complete DMEM/F12 (*C*), or in medium lacking leucine for the indicated time. All values are means ± S.D. ($n = 6$) and were reproduced at least in three independent experiments. * ($p < 0.005$) and ** ($p < 0.001$) indicate a statistically significant difference compared with the cells incubated in complete DMEM/F12.

lator of protein breakdown in muscle cells as starvation for this amino acid is responsible for 30–40% of the increase in protein breakdown generated by total amino acid starvation.

Characterization of the Response to Leucine Availability in C2C12 Myotubes—The role of leucine on muscle protein breakdown was further characterized by: 1) performing short time-course kinetics, 2) studying the effect of leucine re-supplementation, and 3) performing a leucine dose response.

A 3-h time-course experiment showed that leucine starvation induced an acceleration of L-[^{35}S]Met release compared with control, as early as 20–30 min (+7% ($p < 0.05$)) to reach the maximal increase of 11–15% ($p < 0.001$) after 1 h (Fig. 2*A*). To determine whether this response could be reversed, acceleration of protein breakdown was first induced by leucine starvation during 8 h and medium was then replaced by complete medium containing 420 μM leucine (Fig. 2*B*). The accelerated protein breakdown in leucine-replenished cells compared with cells in complete medium was still significant after 15 min but no more after 30 min.

In order to assess whether the effect of leucine starvation was dose-dependent, a time course experiment for protein breakdown with different concentrations of leucine was performed. At 140 or 210 μM leucine, which corresponds to average physiological plasma concentrations of this amino acid (36, 37), no difference in protein breakdown was measured compared with cells cultured with 420 μM of leucine. At 25 μM, leucine increased protein breakdown to almost the same extent as in leucine-starved cells. At 70 or 50 μM leucine, the increase in protein breakdown was always below that measured in leucine-starved cells, but significant with an average of 10% (Fig. 2*C*). Altogether, those results demonstrate that proteolysis in myotubes is sharply regulated by leucine availability in a concentration-dependent manner.

In addition to characterize the effect of leucine on protein breakdown, we measured its effect on protein synthesis by the determination of L-[^{35}S]Met incorporation normalized to protein content (Fig. 2*D*). A time-course experiment indicated that leucine starvation started to decrease global protein synthesis after 3 h to reach a maximum of 20–30% after 8 h or more. The same result was observed with 25 μM leucine in the culture medium, whereas an 8-h exposure to 70 μM leucine did not alter protein synthesis (data not shown).

Characterization of Cellular Response and Intracellular Proteolytic Pathways Involved in the Regulation of Protein Breakdown following Amino Acid or Leucine Starvation—In the liver, it has clearly been shown that amino acids regulate autophagy (10). No study to date has examined whether autophagy could be activated in muscle cells in response to amino acid limitation. Then, the effect of 3-methyladenine, which inhibits the early stages of autophagosome formation (38), was studied (Fig. 3*A*). The presence of the inhibitor in control cells

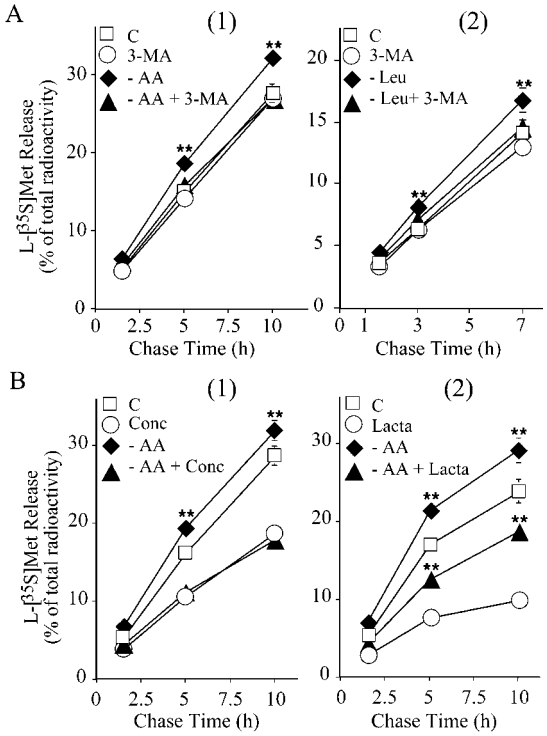
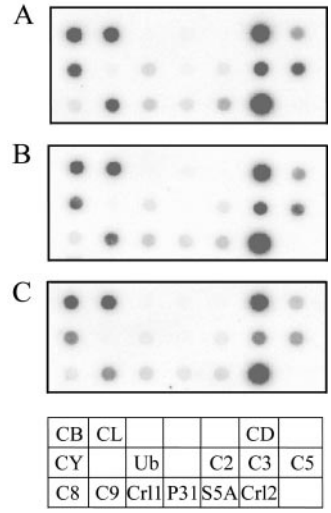


FIG. 3. **Effect of amino acid or leucine starvation on the different proteolytic pathways.** L-[^{35}S]Met release from C2C12 myotubes as an index of total protein breakdown. *A*, effect of the inhibitor of autophagy: 3-methyladenine (*3-MA*, 10 mM). Following protein labeling, myotubes were incubated in complete DMEM/F12 with (○) or without inhibitor (□) and in medium lacking all amino acids (−*AA*, *panel 1*) or leucine (−*Leu*, *panel* 2) with (▲) or without inhibitor (♦). *B*, effect of an inhibitor of lysosome-dependent proteolysis: concanamycin A (*Conc*, 1 μM) (*panel 1*) and of an inhibitor of proteasome-dependent proteolysis: lactacystin (*Lacta*, 2 μM) (*panel 2*). Following protein labeling, myotubes were incubated in complete DMEM/F12 with (○) or without inhibitor (□) and in medium lacking all amino acids with (▲) or without inhibitor (♦). Extracellular trichloroacetic acid-soluble radioactivity was measured during a 10-h time-course experiment (third chase period) as indicated and was expressed as a percentage of the total incorporated L-[^{35}S]Met. All values are means ± S.D. ($n = 6$) and were reproduced at least in three independent experiments. ** ($p < 0.001$) is for a statistically significant difference compared with the cells incubated in complete DMEM/F12.



CB	CL				CD	
CY		Ub		C2	C3	C5
C8	C9	Crl1	P31	S5A	Crl2	

FIG. 4. **Effect of amino acid or leucine starvation on expression of mRNAs encoding different proteolytic actors.** Dot-blots of cDNA targets for some of the proteolytic actors were obtained and hybridized with complex-labeled probes as described under "Experimental Procedures." Complex-labeled probes were generated with 15 μg of total RNA extracted from myotubes cultured 24 h in complete DMEM/F12 (*A*), medium lacking leucine (*B*), or all amino acids (except methionine) (*C*). Hybridization signal intensity was analyzed using Image Quant software and normalized to controls: *A. thaliana* cytochrome c_{554} (*Crl1*) and glyceraldehyde-3-phosphate dehydrogenase (*Crl2*). Blotted cDNAs to perform multiplex messenger RNA assay were as described in the table at *bottom* of figure. Abbreviations used are: *CB*, *CL*, and *CD* for cathepsin B, L, and D; *Cy* for cystatin C; *Ub* for ubiquitin; *C2*, *C3*, *C5*, *C8*, *C9* are for some of the 20 S proteasome subunits; *P31*, *S5A* correspond to some of the 19 S proteasome regulatory subunits.

did not significantly modify the rate of protein breakdown, suggesting a lack or low level of basal autophagy in C2C12 myotubes. When added together with medium lacking all amino acids (Fig. 3*A*, *1*) or leucine (Fig. 3*A*, *2*), 3-methyladenine prevented the protein breakdown from increasing.

As proteins sequestrated by autophagy are degraded in the lysosome, the preferential involvement of lysosome in amino acid regulation of proteolysis was confirmed by using inhibitors of either lysosome- or proteasome-dependent proteolytic pathways. The presence of the lysosome inhibitor concanamycin A in control cells caused a 23–27% ($p < 0.001$) decrease in proteolysis (Fig. 3*B*, *1*). When added together with medium lacking all amino acids, concanamycin A prevented the increase in protein breakdown (Fig. 3*B*, *1*). The presence of lactacystin in control cells also decreased the rate of basal protein breakdown (~45% ($p < 0.001$)) (Fig. 3*B*, *2*). When added together with medium lacking all amino acids (Fig. 3*B*, *2*) or leucine (data not shown), lactacystin did not prevent or reduce the increase of protein breakdown.

Altogether, these results suggest that amino acid withdrawal induces autophagy and that the measured acceleration of protein breakdown is lysosome- but not proteasome-dependent.

Effect of Amino Acid or Leucine Starvation on mRNA Levels of Proteolytic Actors—Because amino acid limitation can affect gene expression at the transcriptional level (39), we studied the effect of amino acids or leucine starvation on mRNA levels of some components of the proteolytic systems. In order to be consistent with experiments measuring the rate of protein degradation, medium lacking all amino acids was supplemented with methionine. As shown in Fig. 4, the increase in the rate of protein breakdown following amino acid starvation is not correlated with an elevation of mRNAs encoding the cysteine proteinases cathepsin B and L or the aspartyl proteinase cathepsin D. In addition, there is no modification in the level of mRNAs encoding ubiquitin or some subunits of the 26 S proteasome. In general, there is a tendency for a slight decrease in mRNA levels for all the tested targets. The overall data suggest that amino acid availability does not exert an effect on proteolysis by modifying gene expression of some major components of the lysosome- and proteasome-dependent proteolytic pathways.

Leucine Starvation Induces Autophagy through an mTOR-independent Signaling Pathway—It has been shown that treatment with rapamycin, an inhibitor of mTOR activity that controls S6 and 4E-BP1 phosphorylation (19, 20), induces autophagy in yeast or in rat hepatocytes, and that amino acids may control autophagy through the regulation of mTOR (21, 22). To determine whether it was also the case in our model of leucine starvation in C2C12 myotubes, the effect of rapamycin treatment was first studied. As shown in Fig. 5*A*, rapamycin induced a 14% ($p < 0.001$) increase in the protein breakdown as compared with control, together with a 20% decrease in protein synthesis, as shown by L-[^{35}S]Met incorporation performed in independent experiments (data not shown). The response was the same with concentrations of rapamycin comprising between 30 and 200 nM (data not shown). When leucine starvation was combined with rapamycin treatment, there was an

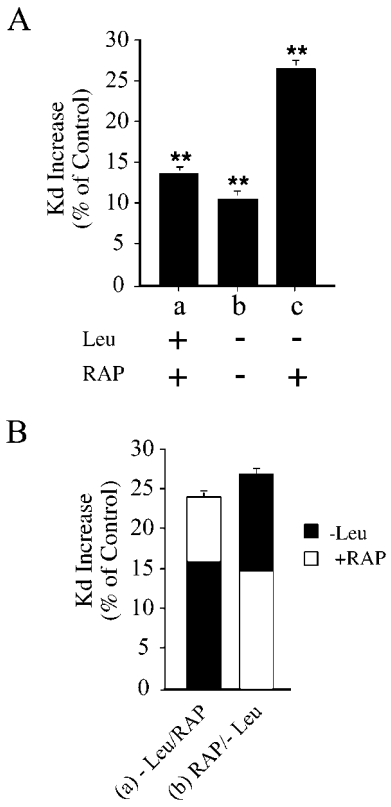


FIG. 5. **Synergetic effect of leucine starvation and rapamycin on protein breakdown in C2C12 myotubes.** *A*, after protein labeling, a 13-h time-course experiment was performed and the K_d increase relative to control in DMEM/F12 was determined. C2C12 myotubes were incubated in complete DMEM/F12, DMEM/F12 + 50 nM rapamycin (*a*), medium lacking leucine (*b*), and finally in medium lacking leucine + 50 nM rapamycin (*c*). *B*, the effect of rapamycin was measured once myotubes have been leucine-starved for 3 h (*a*, *−Leu/RAP*). Alternatively, the effect of leucine starvation was measured once cells had been incubated for 3 h in the presence of 50 nM rapamycin (*b*, *RAP/−Leu*). After protein labeling, a 3-h chase was performed with cells incubated either in complete DMEM/F12 or medium lacking leucine to induce the increase in protein breakdown (*a*, *black bar*). In the next step, half of the cells lacking leucine were incubated with 50 nM rapamycin during a 10-h time-course experiment (*a*, *white bar*). Alternatively, cells were incubated 3 h in complete DMEM/F12 + 50 nM rapamycin (*b*, *white bar*) and in a third step, half of the rapamycin treated cells were leucine-starved (*b*, *black bar*) and catabolism measured through a 10-h time course. In each case (*a* or *b*), the percentage of increase was deduced from the difference between the K_d of the cells in complete DMEM/F12 and the K_d of a given condition after 3 h. After the 10-h time course, the additional effect of rapamycin (*a*) and leucine (*b*) was determined from the difference between the K_d of cells incubated without leucine and those incubated without leucine + rapamycin (*a*) and from the difference between the K_d of cells incubated with rapamycin and those incubated with rapamycin and without leucine (*b*). All values are means ± S.D. ($n = 6$) and were reproduced at least in three independent experiments. ** ($p < 0.001$) is for a statistically significant difference compares with the cells incubated in complete DMEM/F12.

additive effect as a 25% increase in protein breakdown was measured as compared with control (Fig. 5*A*).

This first result suggested the existence of two signaling pathways mediating this response. To verify this hypothesis, two types of experiments were performed (Fig. 5*B*). 1) The effect of rapamycin was measured once myotubes had been leucine-starved for 3 h, and 2) the effect of leucine starvation was measured once cells had been incubated for 3 h in the presence of 50 nM rapamycin. The additive effect of both stimuli was still observed.

The evidence that leucine starvation induces autophagy in muscle cells via an mTOR-independent pathway was further confirmed by the study of the level of phosphorylated S6 ribosomal protein during an 8-h time-course experiment. This is an index of the $p70^{s6k}$ activity, known to be under the control of mTOR (20). The level of the protein was also determined in myotubes starved for all amino acids, except methionine to be consistent with experiments measuring the rate of protein degradation. As shown in Fig. 6, rapamycin treatment induced a loss of the S6-P protein after a 3-h treatment. This confirms that mTOR signaling pathway is effectively inhibited by rapamycin. Therefore, following rapamycin treatment, the effect of leucine starvation on autophagy cannot signal through mTOR. Moreover, a 3-h leucine starvation (Fig. 6) or a 3-h treatment with 70 μM leucine (data not shown) did not modify significantly the level of S6-P compared with controls, indicating that mTOR signaling pathway is not inhibited. However, both treatments induce autophagy (see Fig. 2*C*).

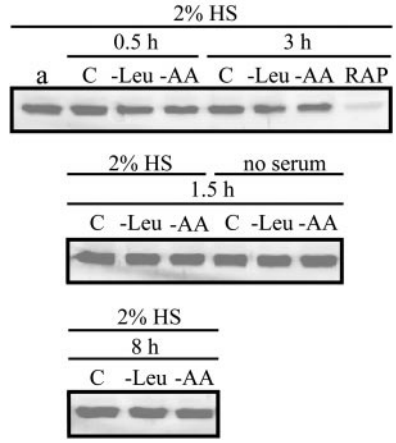


FIG. 6. **Effect of leucine or amino acid starvation and rapamycin on the level of phosphorylated S6.** C2C12 myotubes were incubated during the indicated times with or without serum either in complete DMEM/F12 (*C*), medium lacking leucine (*−Leu*), medium lacking amino acids (except methionine) (*−AA*), complete DMEM/F12 + 50 nM rapamycin (*RAP*). Cell lysates and Western blots were obtained and performed as described under "Experimental Procedures" using antibodies against the S6-P. *Lane a* is for a control with no medium change before protein extraction.

There was no reduction in the level of S6-P after 0.5 or 1.5 h of amino acid or leucine starvation, which excluded a transient effect on $p70^{s6k}$ activity. This result was not a consequence of the presence of serum. Indeed, the removal of horse serum from the medium concomitantly with amino acid deprivation did not modify the level of phosphorylated S6 (Fig. 6). Finally, after 8 h of leucine starvation, when protein synthesis is decreased (see Fig. 2*D*), the amount of S6-P was not different compared with controls. Altogether, these data confirm that leucine starvation regulates autophagy, but also protein synthesis, through a specific signaling pathway not related to mTOR.

DISCUSSION

In skeletal muscle, the existence and activity of two major intracellular proteolytic systems (*i.e.* 26 S proteasome, lysosomal endopeptidases) have been demonstrated. Those degradative pathways, more especially the 26 S proteasome pathway, undergo regulation in response to hormonal variations (12–14, 30). Nutrients can control the level of some circulating hormones and regulate protein turnover. Especially, some amino acids, primarily leucine, can indirectly modulate protein synthesis by acting on insulin secretion (13) and directly by acting on the phosphorylation state of translation initiation factors (17, 18). In the present article, we studied the direct effect of amino acid starvation on skeletal muscle protein breakdown and aimed to characterize cellular responses involved in this process. We specifically studied the effect of leucine, one of the

branched chain amino acids that is oxidized in skeletal muscle and, as stated above, which is known to regulate protein synthesis. We used mouse C2C12 muscle cells, a culture system free from hormone modifications that occur *in vivo*.

This study provides clear evidence that leucine, in addition to regulating protein synthesis, also sharply regulates protein breakdown in a dose-dependent manner. Indeed, leucine starvation accounts for 30–40% of the maximum acceleration of protein breakdown obtained with total amino acid withdrawal. The leucine starvation effect on protein breakdown is maximal and highly significant after 1 h, and leucine re-supplementation for 30 min is sufficient to restore basal levels of breakdown. Alterations in both plasma and muscle amino acid concentrations occur during pathological catabolic states (40, 41), or following modifications of the diet (36, 37, 42). After adaptation to an isocaloric diet, the rat plasma leucine concentration is between 50 and 70 μM compared with 140–200 μM in normal conditions (36, 37). At concentrations between 0 and 70 μM, leucine increases proteolysis in C2C12 myotubes. The direct modulation of protein breakdown by amino acids in muscle cells could be relevant *in vivo* and participate to the transition to a negative nitrogen balance.

In C2C12 myotubes, leucine starvation induces autophagy and as a consequence, an increase in lysosomal-dependent proteolysis, without modification of mRNA levels encoding cathepsins B, L, or D. This implies that amino acids would only regulate the sequestration step of protein substrates for their access to lysosomes. Two main pathways have been described for the targeting to lysosome: 1) sequestration mediated by autophagy in liver, and 2) direct transport, after binding to the soluble receptor hsc73, of proteins carrying the KFERQ motif (43). Using antibodies to KFERQ, proteins carrying this motif were shown to be depleted from liver and heart, but not from skeletal muscle, of fasted rats (44). The latter study and our results support the hypothesis that, following amino acid withdrawal, the proteins would reach the lysosome mainly as a result of the induction of autophagy.

In our model and experimental conditions, the inhibition of the 26 S proteasome does not abolish the increase in the rate of protein breakdown measured after amino acid starvation. In addition, mRNA levels for ubiquitin and some subunits of the proteasome are not affected by total amino acid or leucine depletion. This indicates that amino acids would not exert a direct control of this degradation pathway. Then, as suggested by results on animal models, amino acids would indirectly activate the 26 S proteasome by modification of the hormonal environment and especially by increasing circulating catabolic hormones like glucocorticoids (30).

From recent molecular studies focusing on the direct regulation of protein translation by amino acid availability in different cell lines, it has been shown that amino acids signal to the translational apparatus through the regulation of mTOR (45, 46). Indeed, as observed when mTOR is inhibited by rapamycin (19, 20), withdrawal of some amino acids from nutrient medium results in deactivation of $p70^{s6k}$ and dephosphorylation of 4E-BP1 (17, 18, 45). In our model, we followed global protein synthesis by measurement of L-[^{35}S]Met incorporation and determined the amount of phosphorylated ribosomal S6 protein as an indicator of $p70^{s6k}$ and mTOR activities. The mTOR pathway is submitted to regulation in C2C12 myotubes as rapamycin treatment decreases L-[^{35}S]Met incorporation (data not shown) and inhibits S6 phosphorylation. Nevertheless, leucine withdrawal does not modify S6-P levels through an 8-h time course, although global protein synthesis begins to decrease after 3 h starvation. In L6 myoblasts, it has been shown that leucine depletion for 1 h decreases global protein synthesis by 50% and inhibits $p70^{s6k}$ activity, the latter response being prevented by insulin (17). When C2C12 myotubes were deprived for leucine or all amino acids (except methionine), in the absence of serum, the level of S6-P was not reduced either, which excluded a serum effect in response to amino acids. We also confirmed that there was no transient decrease in S6-P that could have resulted from a transient inactivation of $p70^{s6k}$, as was observed in Jurkat T cells after histidine starvation (46). Those apparent discrepancies could result from the different cell types used and more specifically from the state of differentiation of cells. Unlike myoblasts, myotubes are non-dividing differentiated cells. As occurs in L6 myoblasts, a 70% decrease in the level of S6-P is observed in C2C12 myoblasts cultured in absence of leucine and serum (results not shown). The preferential involvement of mTOR in the regulation of the progression through the cell cycle (47) is consistent with the regulation of this signaling pathway in non-differentiated cells. The conclusion from this study is that in C2C12 myotubes, leucine and most amino acids do not signal to the translational apparatus by regulating mTOR activity.

As is observed for protein synthesis, autophagy is also regulated by protein phosphorylation (22, 48). When protein synthesis is inhibited, it was found that inhibition of autophagic proteolysis following amino acid supplementation, was correlated with the phosphorylation of ribosomal S6 protein in hepatocytes isolated from starved rats (22). The actual concept is that fluxes through the autophagic and synthetic pathways are regulated in an opposite manner with mTOR being the integrator of external signals (hormones or nutrients) and regulating both processes (47). A recent study in yeast clearly demonstrated that mTOR is a negative regulator of autophagy (21). The present work shows that this is also true for C2C12 myotubes, as inhibition of mTOR by rapamycin treatment increases the rate of protein breakdown. However, when mTOR activity is inhibited, leucine starvation further increases protein breakdown. As discussed above, leucine starvation in itself and starvation of all amino acids (except methionine) do not reduce significantly the level of S6-P. Again, the conclusion is that leucine, and probably most amino acids, do not regulate autophagy through mTOR in C2C12 myotubes. Therefore, in this model, the concept of a co-regulation of protein synthesis and degradation by amino acids through the common effector mTOR is not valid. The influence of the state of differentiation on the cellular response to amino acid depletion, as discussed above, is important. However, because mTOR may control liver autophagy (22) contrasting with what occurs in C2C12 myotubes, it suggests that amino acid starvation could turn on distinct signaling pathways in different tissues also characterized by differences in their metabolism.

In conclusion, amino acids and especially leucine can exert a direct effect on muscle protein breakdown. This effect is mediated by the induction of autophagy and increased lysosome-dependent proteolysis with no effect on the 26 S proteasome-dependent proteolytic pathway. We confirm that leucine also regulates protein synthesis in our system, but the primary adaptation to amino acid starvation is induction of autophagy. Although we cannot propose which signaling pathway is involved, we provide clear evidence that leucine effects on both protein synthesis and autophagy in C2C12 myotubes occurs through an mTOR-independent signaling pathway.

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