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# Regulation of various proteolytic pathways by insulin and amino acids in human fibroblasts

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**Abstract** **Intracellular protein degradation is a regulated process with several proteolytic pathways. Although regulation of macroautophagy has been investigated in some detail in hepatocytes and in few other cells, less is known on this regulation in other cells and proteolytic pathways. We show that in human fibroblasts insulin and amino acids reduce protein degradation by different signalling pathways and that this inhibition proceeds in part via the mammalian target of rapamycin, especially with amino acids, which probably increase lysosomal pH. Moreover, the regulatory amino acids (Phe, Arg, Met, Tyr, Trp and Cys) are partially different from other cells. Finally, and in addition to macroautophagy, insulin and amino acids modify, to different extents and sometimes in opposite directions, the activities of other proteolytic pathways.**


*Keywords:* Intracellular protein degradation; Insulin; Amino acids; mTOR; Lysosomes; Proteasomes; Human fibroblasts

## 1. Introduction

Protein degradation and protein synthesis control in a coordinate way the levels of intracellular proteins. The major regulators of protein metabolism are nutrients, such as amino acids, and hormones, such as insulin. Thus, for example, it has been clearly shown that nutrients promote protein synthesis and inhibit protein degradation [1].

In mammalian cells there are about 500 different proteases [2], many of which are within the cells. However, most intracellular proteases produce limited proteolysis and, therefore, there are two main proteolytic pathways in the cells: lysosomes, which are cytoplasmic organelles, and proteasomes, which are multicatalytic proteases localized in the cytosol and in the nucleus. Both pathways are complex and degrade intracellular proteins by different mechanisms. Thus, within lysosomal pathways, macroautophagy is an evolutionary conserved process for bulk proteolysis under nutrient-poor conditions [3]. However, there are also other lysosomal pathways of protein degradation, including microautophagy and chaperone-mediated autophagy [4]. The main non-lysosomal proteolytic pathways involve proteasomes, which degrade proteins by ubiquitin-dependent and -independent mechanisms [5], but other non-lysosomal proteases, including calpains and organellar proteases, can also degrade intracellular proteins to a, comparatively, minor extent [6,7]. Although it is clear that protein degradation is carried out by these various pathways, regulation of macroautophagy by nutrients and hormones has been mainly investigated [8] and it remains to be firmly established if the activity of the other proteolytic pathways can be also regulated.

In addition, most of these studies have been carried out in liver or muscle [8], and it is unknown to what extent there are cell variations in this regulation. Thus, for example, although it has been proposed that the regulation of macroautophagy by amino acids in the liver involves the mammalian target of rapamycin (mTOR) (see [8] and references cited therein), this regulation occurs by a different signalling route in C2C12 myotubes [9].

In this report we have addressed three main questions on the regulation of intracellular protein degradation. First, do the inhibitions by insulin and amino acids overlap? Second, are the regulatory amino acids in other cells the same than in liver or muscle? Third, do insulin and amino acids regulate other proteolytic pathways different from macroautophagy, in particular proteasomes? We have tried to answer these questions using human skin fibroblasts, because their similarity to their in vivo counterparts and also because they can be relatively easily obtained from patients to investigate human pathologies.

## 2. Materials and methods

### 2.1. Materials

Minimum essential medium (MEM), human insulin, 3-methyladenine, $NH_4Cl$, E64d, pepstatin A, cycloheximide, monodansylcadaverine (Fluka), *o*-phthaldialdehyde and the different L-amino acids were purchased from Sigma Chemical Co. MEM amino acids 50×, MEM non-essential amino acids 100×, foetal calf serum, Lysotracker (Molecular Probes) were supplied by Invitrogen Life Technologies. Lactacystin was from Affinity Research Products, leupeptin and *N*-Suc-LLVY-MCA were from Peptide Institute, Inc. and rapamycin was from Calbiochem. Most of the antibodies used have been described before [10,11]. In addition, phospho-specific antibodies which recognize 4E-BP1 (T37/T46) and p70S6K (T389), as well as their non-phospho-specific antibodies, were from Cell Signalling and the mouse monoclonal antibody to LC3 (clone 5F10) was from Nanotools. Radioisotopes were obtained from Amersham Pharmacia Biotech. Other reagents were of the best analytical quality available.

### 2.2. Cell culture

Normal human skin fibroblasts were obtained from the Coriell Institute for Medical Research (Camden, NJ, USA). Cells were grown at 37 °C in a humidified atmosphere of 5% (v/v) $CO_2$/air in MEM with

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Earle's salts, 2 mM L-glutamine, 1× MEM amino acids, 1× MEM non-essential amino acids, 1× MEM vitamins, 100 U/ml penicillin, 100 μg/ml streptomycin (fibroblasts' growth medium) and containing or not 20% foetal calf serum. Cell viability and growth curves were determined in parallel for each culture. All experiments were performed at 14–20 passage number to avoid culture aging effects.

### 2.3. Measurements of intracellular protein degradation

Fibroblasts were incubated for 48 h in fresh full medium with 5 μCi/ml [$^3$H]valine or, in some experiments (e.g. those to analyze the effects of valine), with 5 μCi/ml [$^3$H]leucine, followed by a 24 h chase in fresh full medium containing 10 mM L-valine (or 2 mM L-leucine) to degrade short-lived proteins [12]. Then, and to measure degradation of long-lived proteins, all cultures were incubated for 4 h in Krebs–Henseleit medium (118.4 mM NaCl, 4.75 mM KCl, 1.19 mM $KH_2PO_4$, 2.54 mM $MgSO_4$, 2.44 mM $CaCl_2 \cdot 2H_2O$, 28.6 mM $NaHCO_3$, 10 mM glucose) with 10 mM Hepes, pH 7.4, or in fibroblasts' growth medium, containing 10 mM L-valine (or 2 mM L-leucine) and the indicated additions. Protein degradation was analyzed 1 h later to ensure maximal effects of the various additions and for only 3 h to avoid possible secondary effects, and was calculated at intervals of 1.5 h by measuring the net release of trichloroacetic acid-soluble radioactivity from the labelled cells into the culture medium and expressed as percentage protein degraded in 1.5 h. The contribution of the various proteolytic pathways was calculated as previously described [13].

### 2.4. General procedures

The immunoblotting procedure has been described [13]. With phospho-specific antibodies, they were always used in the first round of immunoblotting. After treating the membrane with stripping buffer (0.1 M glycine, pH 2.3), it was probed using the antibodies that recognize the total amount of the specific protein of interest. Immunofluorescence microscopy and electron microscopic morphometry were carried out as detailed in [14,15], respectively. Intracellular leucine analysis was performed essentially as in [16], after derivatization of amino acids with *o*-phthaldialdehyde, on a reversed-phase C18 column (3.9 × 150 mm) and using a 717 (2 × 515) HPLC with a 2475 Multi λ Fluorescence Detector (Waters Corporation). Protein concentration was measured with sodium deoxycholate, using bovine serum albumin as the standard. The chymotrypsin-like activity of proteasomes in crude cell extracts was determined, using *N*-Suc-LLVY-MCA, as the difference between total activity and the remaining activity in the presence of 20 μM lactacystin [17]. Intracellular monodansylcadaverine was measured (excitation wavelength 380 nm, emission wavelength 525 nm) in a Spectra Max M5 microplate reader (Molecular Devices) and expressed as % specific activity (arbitrary units) as described [18]. Comparisons between different conditions were by Student's *t*-test. $P < 0.05$ was taken as significant.

## 3. Results

### 3.1. Insulin and amino acids inhibit the degradation of long-lived proteins in human fibroblasts in an additive way

In previous experiments, the effects of various concentrations of insulin and amino acids on protein degradation in human fibroblasts incubated in Krebs–Henseleit medium for 4 h or less were analyzed (data not shown). The highest inhibition (Fig. 1A) was found at $10^{-6}$ M insulin and at an amino acid concentration two times that present in the growth medium (29% and 34% inhibitions, respectively). The inhibition produced when insulin and amino acids were added together (63%) was clearly additive, indicating that they probably act through different signalling pathways. When the Krebs–Henseleit medium was replaced by fibroblasts' growth medium without (SW) or with (Med) serum, the inhibitions (40% and 69%, respectively) were only slightly below those obtained in Krebs–Henseleit medium with amino acids alone or plus insulin, respectively (see Fig. 1A), indicating that both are the main

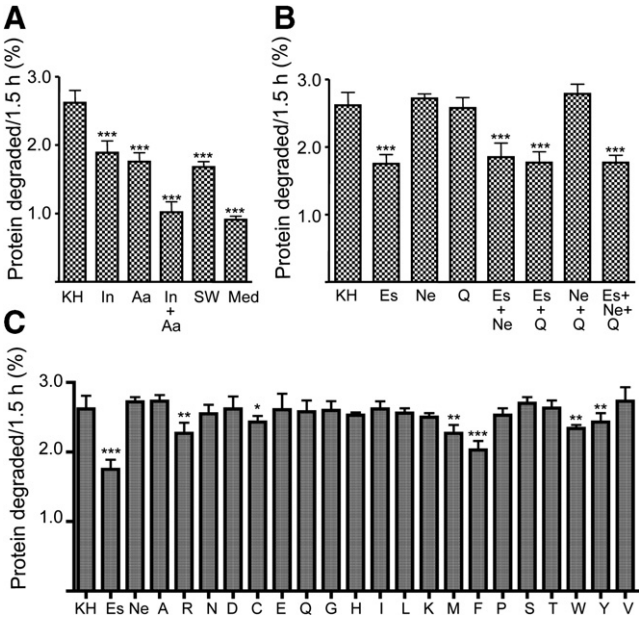


Fig. 1. Effects of insulin and/or amino acids on the degradation of long-lived proteins in human fibroblasts. Exponentially growing human fibroblasts were metabolically labelled with [$^3$H]valine or [$^3$H]leucine for 48 h, chased for 24 h and then switched to fibroblasts' growth medium without (SW) or with (Med) 20% foetal calf serum or to Krebs–Henseleit medium (KH) with the following additions (final concentrations): in A, $10^{-6}$ M insulin (In), and/or amino acids (Aa) at two times the concentration present in the fibroblasts' growth medium; in B, MEM amino acids (Es) and/or non-essential amino acids (Ne) (see text) at two times the concentration present in the fibroblasts' growth medium, and/or 2 mM glutamine; and in C, each amino acid at 2 mM concentration. Protein degradation was measured, as described in Section 2, after 1 h of the indicated additions and up to 4 h at 1.5 h intervals (i.e. at 2.5 and 4 h chase). Results are the mean and S.D. from eight to fifteen separate experiments with duplicate samples. Stars indicate statistically significant differences from KH values at $^*P < 0.05$, $^{**}P < 0.005$, and $^{***}P < 0.0005$.

inhibitors of intracellular protein degradation in human fibroblasts under these conditions.

We also investigated whether or not these inhibitions proceed *via* the mammalian target of rapamycin (mTOR) signalling pathway (Fig. 2). Both insulin and amino acids increased the phosphorylation of two substrates of mTOR, 4E-binding protein 1 (4E-BP1, Fig. 2A) and ribosomal S6 kinase (p70S6K, Fig. 2B), in a rapamycin sensitive manner. Also, the inhibition of protein degradation by insulin and especially by amino acids was, at least in part (20 ± 3% and 52 ± 4%, respectively), sensitive to this mTOR inhibitor and these inhibitions were clearly additive (Fig. 2C).

### 3.2. The regulatory amino acids in human fibroblasts are partially different from other cell types

To investigate which amino acids were regulatory, we first tested separately the three available commercial solutions (see Section 2): a mixture of amino acids which are required for growth (MEM amino acids: Arg, Cys, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, Tyr, Val), or of amino acids which can be synthesized by the cells (Non-essential amino acids: Ala, Asn, Asp, Glu, Gly, Pro, Ser) and Gln. As shown in Fig. 1B, while non-essential amino acids (Ne) and Gln had no significant inhibitory effect, MEM amino acids (Es) produced exactly

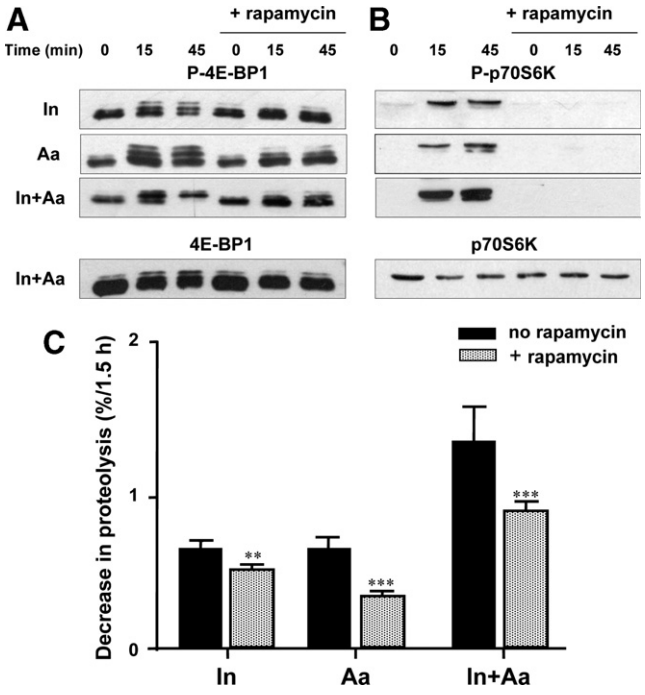


Fig. 2. Implication of mTOR in the inhibition of intracellular protein degradation by insulin and/or amino acids. (A and B) Cells were incubated for the indicated times in Krebs–Henseleit medium, in the absence or in the presence of 200 nM rapamycin, and with insulin (In) and/or amino acids (Aa) as shown. Cell lysates were analyzed by Western blotting using phospho-specific antibodies for 4E-BP1 (P-4E-BP1) (A) and p70S6K (P-p70S6K) (B) or antibodies that recognize the total amount of the protein (4E-BP1 or p70S6K). (C) Human fibroblasts were radioactively labelled and protein degradation, with or without insulin and/or amino acids and in the presence or not of 200 nM rapamycin as shown, was measured as described in the legend to Fig. 1. Results are presented as the decrease in the percentage of the labelled protein which is degraded in 1.5 h in Krebs–Henseleit medium in the presence of insulin and/or amino acids as indicated and with (gray bars) or without (black bars) rapamycin. Each value is the mean and S.D. from at least six separate experiments with duplicate samples. Differences from incubation values without rapamycin were found to be statistically significant at $^{**}P < 0.005$ or $^{***}P < 0.0005$.

the same effect than all amino acids (Es + Ne + Q). Therefore, we considered the possibility that protein synthesis was required for this inhibition. As shown in Fig. 3A and B, although cycloheximide, which at the concentration here employed inhibits protein synthesis by 97.1 ± 0.4%, reduced the cellular fractional volume of autophagic vacuoles, amino acids further decreased this parameter. Moreover, we found in pulse-chase experiments that, while cycloheximide partially abolishes intracellular protein degradation (36 ± 2%), the percentage of protein degraded which is inhibited by amino acids (or insulin) was the same with or without protein synthesis (Fig. 3C). Also, the protein degraded by macroautophagy in 1.5 h which is sensitive to insulin or amino acids was not significantly different in the absence (0.72 ± 0.10% or 0.82 ± 0.15%, respectively) or in the presence (0.66 ± 0.05% or 0.79 ± 0.10%, respectively) of cycloheximide. Finally, the ratios of the cytoplasmic (LC3-I) to the membrane bound (LC3-II) forms of endogenous LC3, after inhibition of lysosomal proteases [19], were essentially comparable under the various conditions in control cells and in cells treated with cycloheximide at two different concentrations (Fig. 3D). Thus, it appears that amino acids inhibit protein degradation by a process which does not require protein synthesis.

When the individual amino acids were tested separately (Fig. 1C) at 2 mM concentration, it was found that only Arg, Met, Phe, Trp, Tyr and, to a lesser extent, Cys significantly inhibited protein degradation. The inhibitory effects of the individual amino acids varied, being Phe the most effective inhibitor (20%), whereas Cys was the less effective (6%). Surprisingly, Leu and Gln, which are important regulators of protein degradation in other cell types, had no significant effects in human fibroblasts (Fig. 1C), even when added together (data not shown). In the case of leucine, the possibility that its intracellular concentration in human fibroblasts may be very low seems unlikely, since after addition of 1, 2 and 10 mM leucine to the Krebs–Henseleit medium, the intracellular concentration of leucine in human fibroblasts, measured by HPLC, rised quickly from 6 ± 1 to 913 ± 100, 1385 ± 326 and 2567 ± 1273 nmols/g cell protein, respectively. Similar measurements carried out in parallel in C2C12 myotubes (where leucine inhibits protein degradation) indicated even lower intracellular leucine concentrations: 6 ± 1 (without leucine), 95 ± 24 (1 mM leucine), 242 ± 10 (2 mM leucine) and 981 ± 150 (10 mM leucine) nmols/g cell protein. Therefore, it appears that the regulatory amino acids vary in different cell types.

### 3.3. Insulin and amino acids regulate the activity of various proteolytic pathways in human fibroblasts

To investigate if only macroautophagy was affected by amino acids and insulin, we calculated first, by previously described procedures [13], the contribution of various pathways to intracellular protein degradation in Krebs–Henseleit medium and, then, we analyzed the variations produced when insulin and/or amino acids were added. Fig. 4 shows that most protein degradation in Krebs–Henseleit medium occurs by macroautophagy and proteasomes (63% and 25%, respectively). Insulin and amino acids, inhibited quite effectively (43% and 48%, respectively, and 92% both together) the degradation of long-lived proteins by macroautophagy (Fig. 4A) and also decreased (about 42% and 26%, respectively, and 66% when used together) the amount of proteins degraded by proteasomes (Fig. 4B). In contrast, insulin and especially amino acids apparently stimulated (about two and three times, respectively) the degradation of proteins by lysosomal pathways different from macroautophagy (other lysosomal, Fig. 4C). Finally, insulin stimulated (1.6 times) the degradation of non-lysosomal pathways which are different from proteasomes, while amino acids slightly inhibited (31%) this degradation (other non-lysosomal, Fig. 4D). Therefore, it appears that the various proteolytic pathways are differently regulated. Essentially the same effects to those described for amino acids alone or plus insulin, respectively, were found when using, as controls, fibroblasts' growth medium without (SW) or with (Med) serum (Fig. 4). In all these cases, the effects of insulin and amino acids were fully additive, in agreement with the results when analysing total protein degradation (see Fig. 1A).

We next investigated the effects of insulin and of amino acids on several lysosomal and proteasomal parameters. Under the conditions of these experiments, neither insulin nor amino acids produced variations in the levels of some lysosomal marker proteins, such as lamp-1, lamp-2 and cathepsin D (as studied by Western blot and immunofluorescence) or in the

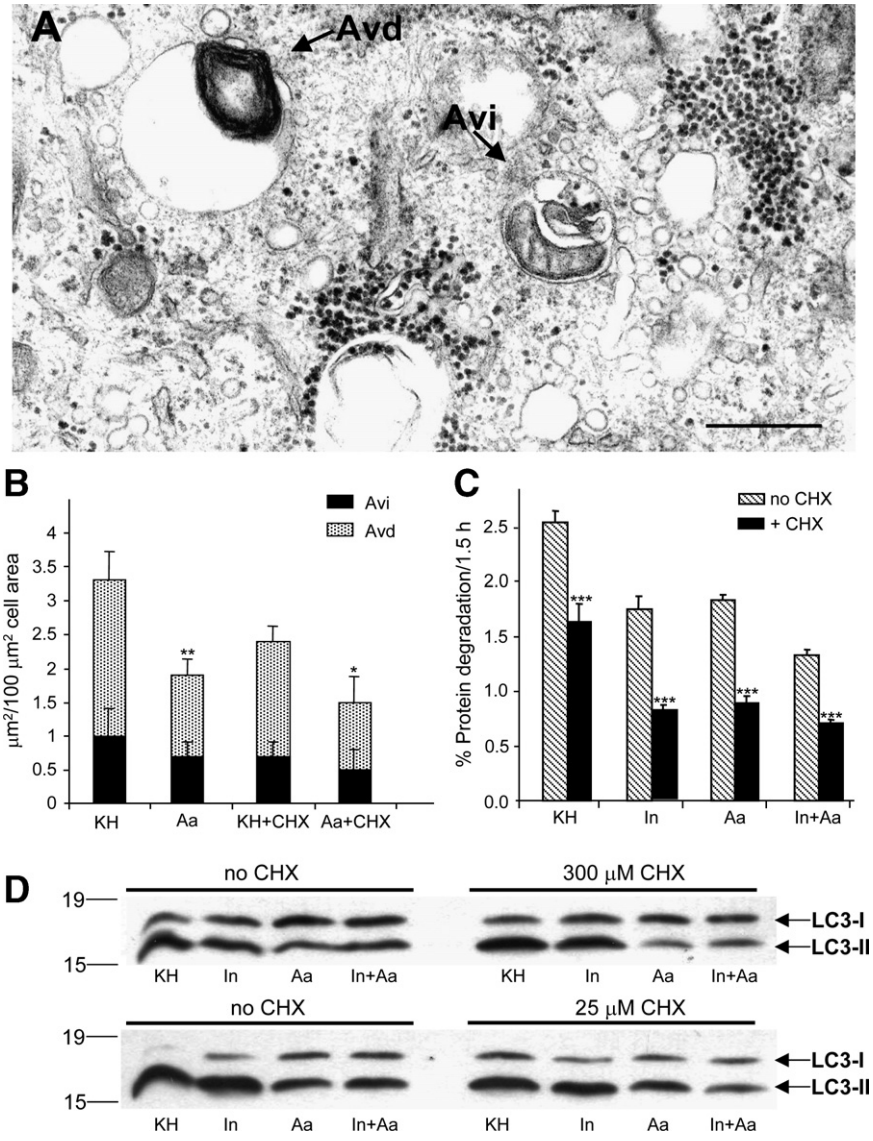


Fig. 3. Inhibition of protein synthesis does not affect the regulation of protein degradation by amino acids in human fibroblasts. (A) Representative electron micrograph of the cells incubated in Krebs–Henseleit medium showing early (Avi) and late (Avd) autophagic vacuoles. Bar: 0.5 μm. (B) The amounts of early (Avi) and late (Avd) autophagic vacuoles in cells incubated in Krebs–Henseleit medium in the presence (Aa) or not (KH) of amino acids and treated or not with 100 μg/ml cycloheximide (CHX) were measured by electron microscopic morphometry. Each value is the mean and S.D. from two separate experiments, with two different blocks analyzed in each. Amino acids data, with or without cycloheximide, were compared with the corresponding values without amino acids and differences were found to be statistically significant at $^{*}P < 0.05$ and $^{**}P < 0.005$. (C) Human fibroblasts were radioactively labelled and incubated in Krebs–Henseleit medium with the indicated additions, in the presence (+CHX) or not (no CHX) of 100 μg/ml cycloheximide and protein degradation was measured as described in the legend of Fig. 1. Each value is the mean and S.D. from two separate experiments with duplicate samples. Differences from incubation values without cycloheximide were found to be statistically significant at $^{***}P < 0.0005$. (D) Western blotting of the autophagic marker protein LC3 in cells incubated in Krebs–Henseleit medium with the indicated additions (KH, no additions; In, insulin; Aa, amino acids), in the presence of 10 μg/ml each of E64d and pepstatin A, and without (no CHX) or with the indicated concentration of cycloheximide (CHX). The positions of LC3-I and LC3-II are indicated on the right and molecular-mass markers and their size in kDa on the left.

activities of cathepsins B, L or D (data not shown). Both insulin and amino acids reduced the lysosomal fractional volume (by 55 ± 12% and 62 ± 9%, respectively), but, although when insulin and amino acids were used together this decrease was slightly higher (78 ± 6%) than when added separately, it was not additive, in contrast with the inhibitions of intracellular protein degradation. The main differences that we found in the effects of amino acids and insulin on lysosomes were two: amino acids, but not insulin, produce a decrease in the mean size of lysosomes (52 ± 27%, differences from control or insulin values significant at $P < 0.0005$) and in the fluorescence intensity of Lysotracker (data not shown) and monodansylcadaverine (Fig. 5). Since both stains accumulate in acidic organelles, these differences may be related to an increase in lysosomal pH produced by amino acids (compare Fig. 5B and C and data in E).

Insulin and amino acids also affect proteasomes, but, under the conditions of the experiments (4 h incubations), they do not modify in an ostensible way the levels of various subunits of the 20S proteasomes and their regulatory complexes (C9, Z,

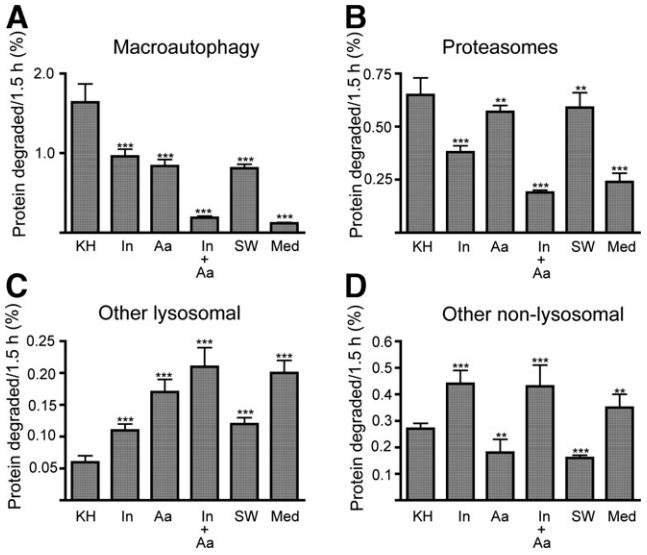


Fig. 4. Effects of insulin and/or amino acids on the activities of the various pathways of protein degradation. Human fibroblasts were radioactively labelled and incubated in Krebs–Henseleit medium with the indicated additions as described in the legend of Fig. 1. The contribution of macroautophagy (A), proteasomes (B), lysosomal pathways different from macroautophagy (C, other lysosomal) and non-lysosomal pathways different from proteasomes (D, other non-lysosomal) to the degradation of proteins was calculated and results are presented as percentage of the labelled protein which is degraded in 1.5 h by the various protein degradation pathways under the different conditions. Results are the mean and S.D. from eight to fifteen separate experiments with duplicate samples.

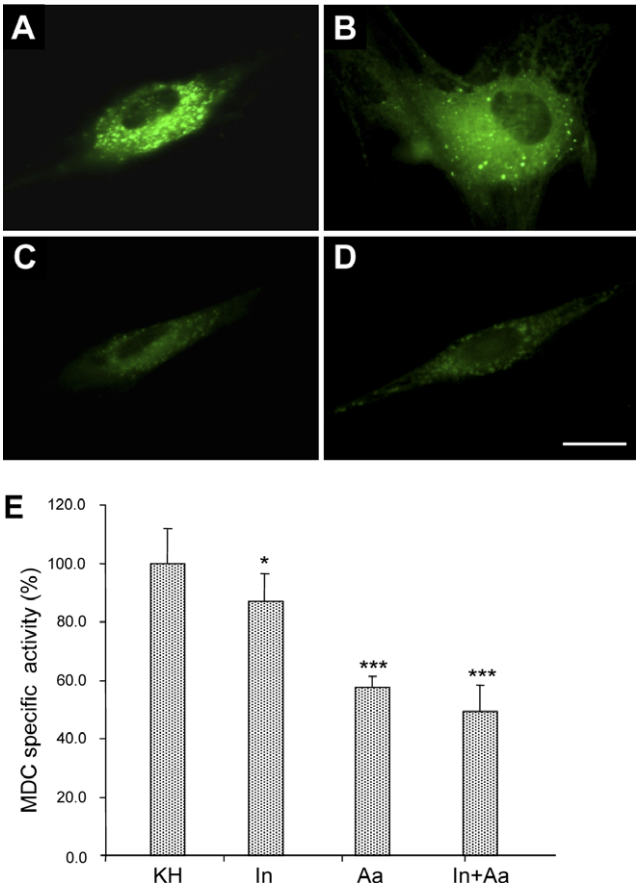


Fig. 5. Effects of insulin and/or amino acids on monodansylcadaverine staining of lysosomes. Cells were incubated in Krebs–Henseleit medium (KH) for 90 min without (A) or with insulin (B), amino acids (C) or insulin plus amino acids (D). Then, 50 μM monodansylcadaverine was added for 10 min at 37 °C and, after three washes with ice-cold phosphate buffered saline, the cells were immediately observed by fluorescence microscopy using an inverted microscope. Bar: 10 μm. In (E), intracellular monodansylcadaverine (MDC) was measured in KH without or with insulin (In) and/or amino acids (Aa) as described in Section 2. Data represent the mean and S.D. of four (In) or six (the rest) different experiments. Differences from KH values were found to be statistically significant at $^{*}P < 0.05$ or $^{***}P < 0.0005$.

LMP2, α-PA28, S1, p45) or the nuclear vs cytosolic localization of proteasomes (data not shown). However, they produce a decrease in the chymotrypsin-like activity of proteasomes (about 9 ± 6%, 13 ± 8% and 23 ± 6% in the presence of insulin, amino acids, and both together, differences from control values significant at $P < 0.05$, $P < 0.005$ and $P < 0.0005$, respectively) as well as a slight decrease in the amount of polyubiquitinated proteins, which is more evident with insulin (Fig. 6).

## 4. Discussion

Variations in the concentrations of amino acids and insulin regulate both protein synthesis and protein degradation in some cell types (reviewed in [1]). In human fibroblasts, insulin and amino acids inhibit protein degradation independently of each other, since, when added together, these effects were additive. Therefore, apparently in these cells, the signalling pathways by which they control intracellular protein degradation do not overlap, although it is usually believed, at least in certain cell types, that both converge at the level of mTOR [8]. Alternatively, it is possible that amino acids and/or insulin modify the action of the other regulator, but since the additivity, even when analysing separately different proteolytic pathways, is quite accurate, the first possibility appears more likely. In fact, it has been proposed, in isolated rat hepatocytes, that insulin and amino acids control macroautophagy through different signalling pathways in relation to mTOR [20], although this is not the prevalent point of view in those cells (see e.g. [8]). In human fibroblasts, and on the basis of the observed effects with rapamycin, mTOR appears to be clearly implicated in the amino acid-dependent regulation of protein breakdown. This is in agreement with initial observations in hepatocytes of a correlation of the inhibition of protein degradation by amino acids and phosphorylation of the small ribosomal subunit S6 [21] and with later results in other cells (reviewed in [8,22]). Moreover, mTOR appears also to be implicated, but to a lesser extent, in the insulin-dependent regulation. However, in both cases other signalling pathways different from mTOR should also contribute to this regulation, because the inhibitions of protein degradation are only partially sensitive to rapamycin, particularly in the case of insulin (see Fig. 2C). In fact, additional mTOR-independent pathways have been postulated for the regulation of macroautophagy [23].

In human fibroblasts there are differences with other cell types in the nature of the regulatory amino acids of intracellular protein degradation. Thus, in rat liver, eight amino acids (Leu, Gln, Tyr, Phe, Pro, Met, Trp and His) have a direct

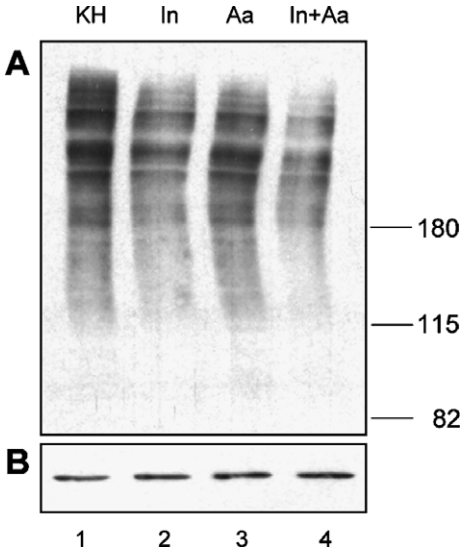


Fig. 6. Insulin and, to a lesser extent, amino acids decrease the amount of polyubiquitinated proteins. Extracts (75 μg protein) of cells incubated for 30 min in Krebs–Henseleit medium (KH) without or with insulin (In) and/or amino acids (Aa) were analyzed in a Western blot using antibodies which recognize ubiquitinated proteins (A) or actin (B) which served as a loading control. The positions of molecular-mass markers and their size in kDa are indicated on the right.

regulatory potential (reviewed in [24]). However the regulatory amino acids in human fibroblasts are partially different, since Arg, and perhaps Cys, are also regulatory, while Leu, Gln, Pro and His are not. This is particularly important with Leu, since it has been found to inhibit protein degradation, not only in liver but also in skeletal muscle and adipocytes (reviewed in [8,22,25]), and it has been considered as the main signal for amino acid availability [1,26]. In addition, it has been proposed that Leu is recognized at the plasma membrane, indicating the possible existence of an amino acid receptor/sensor for subsequent intracellular signalling (see e.g. [24]). Since Leu accumulates within human fibroblasts and has apparently no effect on intracellular protein degradation in these cells, the activity of this hypothetical receptor may be cell-type dependent. Also, it has been shown that the activities of the enzymes which catabolyze leucine and the other branched chain amino acids considerably differ among various species and tissues [27,28]. Therefore, cell-dependent divergences in regulation may occur, probably because protein degradation serves distinct physiological roles in different cell types, and the possibility that amino acids regulate intracellular protein degradation by products of their cell-specific metabolism can not be excluded. However, this appears not to be the case for some of the observed Leu effects in other cells [29].

Among the various cellular proteolytic pathways, macroautophagy is considered a physiologically regulated mechanism of bulk protein degradation, which modifies its activity in response to multiple signals [3,8]. In contrast, the fine degradation of specific proteins is mainly controlled by the ubiquitin-proteasome pathway [30] and, therefore, it is usually believed that regulation of this activity mainly occurs at the level of the substrate proteins. However, the enhanced proteolysis produced in various pathological states in muscle cells has been considered to be primarily due to activation of the ubiquitin-proteasome system [31], and it has been also shown that insulin and amino acids can inhibit proteasomes [32,33]. Here we show that insulin inhibits, to about the same extent, macroautophagy and proteasomes, whereas amino acids are more effective with macroautophagy. Therefore, it appears that proteasomes and macroautophagy, the main proteolytic pathways in mammalian cells, can be regulated by similar signals. In this regard, it has been recently shown in C2C12 myotubes that amino acids act additively with insulin to down regulate the ubiquitin-proteasome pathway [34], in agreement with our observations.

The inhibition of protein degradation produced by amino acids, and also by insulin, does not require protein synthesis. The most clear and common effects on lysosomes of amino acids and insulin are a decrease in their fractional volume in a non-additive way, without modifying the levels or activities of some major lysosomal components, and it appears also that, as expected, there are some differences in their specific effects on lysosomes. In particular, the fluorescence intensity of Lysotracker (and also of monodansylcadaverine, because its staining derives from both interaction with autophagic membrane lipids and accumulation in acidic compartments [35]), may explain, at least in part, the additive effects of insulin and amino acids on macroautophagic activity, with amino acids slightly increasing lysosomal pH, while insulin inhibiting autophagosome formation. However, additional work would be required to test this possibility and to precisely work out the change in lysosomal pH produced by amino acids. In this regard, we have recently found, in a proteomics 2D-DIGE approach, that the levels of various subunits (at least, subunits A, B, C and E) of the vacuolar ATPase (which acidifies lysosomes) decrease in lysosomal membranes isolated from cells incubated in the presence of amino acids, when compared to cells incubated in their absence (data not shown).

With proteasomes, the decreased activity of this pathway is not associated with variations in localization or in the levels of proteasomal subunits and only with a minor reduction in the amount of polyubiquitinated proteins. Amino acids and insulin also inhibit the chymotrypsin activity of proteasomes, but only very slightly and, in contrast with the effects in intact cells, the former are more effective than the latter. Therefore, some additional mechanism, not yet identified, should contribute to the inhibition of the activity of proteasomes in the cells, especially in the case of insulin.

In addition to macroautophagy and proteasomes, there are other minor proteolytic pathways operating in the cells [4,6,7]. Here we show that insulin activates these pathways whereas amino acids have a differential effect, strongly activating lysosomal pathways different from macroautophagy but slightly inhibiting the non-lysosomal pathways different from proteasomes. The physiological meaning of the stimulation of proteolysis in these systems, which is probably produced by the different regulation of at least some of them (e.g. [36,37]), is unclear. There are several examples of collaboration of proteolytic systems (proteasomes, macroautophagy, chaperone-mediated autophagy and calpains, shortly reviewed in [38]) and a decreased activity of one of those can be compensated by activation of other (see e.g. [39]). Here, probably because, compared to proteasomes and macroautophagy, the activities of the lysosomal pathways different from macroautophagy and of the non-lysosomal pathways different from proteasomes are low (compare in Fig. 4 the absolute values of C and D with those in A and B in Krebs–Henseleit medium),

they could amend the decrease or even loss of some minor but important function of proteasomes and/or macroautophagy, when the general activities of these pathways are inhibited. This remains to be investigated.

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