Role of AMP-activated protein kinase in autophagy and proteasome function

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ABSTRACT

In this work, we have examined the possible role of AMP-activated protein kinase (a key energy sensor) in regulating intracellular protein degradation. We have found that AICAR, a known activator of AMPK, has a dual effect. On one hand, it inhibits autophagy by a mechanism independent of AMPK activity; AICAR decreases class III PI3-kinase binding to beclin-1 and this effect counteracts and reverses the known positive effect of AMPK activity on autophagy. On the other hand, AICAR inhibits the proteasomal degradation of proteins by an AMPK-dependent mechanism. This is a novel function of AMPK that allows the regulation of proteasomal activity under conditions of energy demand.

Key words: AICAR, AMPK, proteasome, PI3-kinase, protein degradation, autophagy.

INTRODUCTION

Intracellular protein degradation is a catabolic process by which all intracellular proteins in prokaryotic and eukaryotic cells are continuously broken down [1]. The major sites of intracellular protein degradation in mammalian cells are the proteasomes and the lysosomes. Both degrade proteins by energy-consuming processes requiring ATP ([2], [3]). Therefore, intracellular protein degradation is a highly energy-demanding process and, thus, it is expected that under conditions of energy depletion this process should be tightly regulated. In fact, this is the case for the proteasomal protein degradation pathway, where it has been found that prolonged starvation or severe heat shock conditions promote proteasome dissociation, resulting in a rapid decline in intracellular proteolysis ([4], [5]). However this rationale is not that evident for lysosomal protein degradation, since it has been reported that this process is

activated by various stress situations, including nutrient depletion. This may be explained because under these stress conditions, autophagy provides constituents required to maintain the essential metabolism for survival [6].

AMP-activated protein kinase (AMPK) is a conserved sensor of cellular energy which protects cells by acting as a low-fuel warning system. AMPK is a heterotrimer composed of three different subunits, i.e. α , β and γ . AMPK α is the catalytic subunit (with two isoforms $\alpha1$ and $\alpha2$), whereas AMPK β (with two isoforms $\beta1$ and $\beta2$) and AMPK γ (with three isoforms $\gamma1$, $\gamma2$ and $\gamma3$) play regulatory roles. The activity of AMPK is regulated by phosphorylation of the catalytic alpha subunit at Thr172 by an upstream kinase, sharing LKB1 and CaMKK β this role (see [7] for review). Since AMPK is a sensor of cellular energy, it seems plausible that AMPK may play a role in the regulation of intracellular protein degradation. In fact, it is known that the yeast orthologue of AMPK (Snf1) stimulates autophagy in yeast [8]. However, in isolated hepatocytes, initial reports using 5-aminoimidazole-4-carboxamide 1- β -D-ribofuranoside (AICAR) as an activator of AMPK [9], suggested that AMPK had the opposite effect [10]. Nevertheless, more recent reports clearly indicate that AMPK, like its yeast Snf1 counterpart, has a positive effect on autophagy ([11], [12], [13]).

The purpose of this work was to examine the effect of AMPK on the main proteolytic pathways in human fibroblasts. To this end, we used AICAR as an activator of AMPK function and found that this compound can prevent intracellular protein degradation by two mechanisms, one which decreases the amount of autophagic vacuoles by interfering with the function of class III PI3-kinase, and another preventing proteasome function. We also demonstrate that the effect of AICAR on lysosomes is independent of AMPK action, whereas its effect on proteasomes depends on AMPK activity.

MATERIALS AND METHODS

Cell culture

Healthy human skin fibroblasts (3349B) were obtained from the Coriell Institute for Medical Research (Camden, NJ) and grown in MEM (Sigma, St. Louis, MO) with Earle's salts as described [14]. AMPK α subunit double knockout mouse embryonic fibroblasts (MEFs) ($\alpha 1^{-/-}$ and $\alpha 2^{-/-}$) and wild type controls were grown as described previously [15].

Adenovirus infection

Adenovirus encoding a dominant negative form of AMPK (Ad-DN-AMPK; AMPK $\alpha 1$ D157A), a constitutively active AMPK form (Ad-CA-AMPK; AMPK $\alpha 1_{1-312}$ T172D), or encoding GFP (Ad-GFP), all kindly provided by Dr. P. Ferré and Dr. F. Foulle (INSERM Unit 671, Université Paris 6, Centre de Recherches Biomedicales des Cordeliers, Paris, France), were propagated as described in [16].

Measurements of intracellular protein degradation by pulse-chase experiments.

Fibroblasts were incubated for 48h in fresh complete medium with 5 μ Ci/ml [3 H]valine (Amersham Biotech; Piscataway, NJ) and then chased for 24h in fresh complete medium containing 10 mM L-valine. To measure protein degradation, cultures were incubated for 4h in Krebs-Henseleit medium and protein degradation was analyzed as in [14].

Immunoblot analysis.

Cell lysates were prepared as in [14]. Sixty μ g of total protein from cell lysates were analyzed by SDS-PAGE and western blotting using the appropriate antibodies: anti-phospho-T172-AMPK, anti-AMPK α , anti-phospho-S473-Akt, anti-Akt, were from Cell Signaling Technology (Beverly, MA); anti-PI3-kinase class III and anti-GFP were from Abcam (Cambridge, UK); monoclonal anti-beclin (clone12B4) and anti-LC3 (clone 5F10) were from Nanotools (Teningen, GER). The secondary antibodies coupled to horseradish peroxidase and the anti-actin antibodies were from Sigma (St. Louis, MO).

Electron microscopy.

Preparation of samples for electron microscopy and morphometric measurements were carried out as detailed in [17]. For each condition, about thirty different electron micrographs from four different samples were analyzed.

Determination of proteasome activity.

Wild type and AMPK α subunit double knockout (α 1-/- and α 2-/-) MEFs (35,000 cells) were cultivated in DMEM medium (Lonza Bioscience, Barcelona). Then, they were incubated for 4 h in Krebs-Henseleit medium containing or not 0.5 mM AICAR, 2 mM metformin or 20 μ M lactacystin. Finally, the chymotrypsin-like activity of proteasomes was measured using the Proteasome-Glo Cell-Based Assay with N-Suc-LLVY-aminoluciferin as substrate (Promega, Madison, WI), following the manufacturer's instructions. Luminiscence was measured in a Spectra Max M5 microplate reader (Molecular Devices, Sunnyvale, CA). Results were referred to the activity found in wild type MEFs cells growing in Krebs-Henseleit medium without additions.

Statistical data analysis.

Data are expressed as means $\pm$ standard deviation (SD). Statistical significance of differences between the groups was evaluated by a paired Student's t test with two-tailed distribution. The significance has been considered at * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$, as indicated in each case.

RESULTS**1) AICAR treatment prevents protein degradation by both proteasomal- and lysosomal-independent mechanisms.**

To explore a possible role of AMPK in protein degradation we measured the degradation of radiolabeled proteins in human fibroblasts under conditions of AMPK activation. To differentiate protein degradation mediated by proteasomal and lysosomal pathways, we treated the cells with either lactacystin (a proteasomal inhibitor) or a mixture of ammonium chloride and leupeptin (NH_4/Leup ; which inhibit lysosomal-dependent degradation). As shown in Fig. 1, lactacystin inhibited about 1/3 of total protein degradation, whereas NH_4/Leup made a reduction of around 2/3. The combined treatment of lactacystin plus NH_4/Leup prevented almost completely protein degradation. Then, we treated the cells with AICAR (an AMPK activator; [9]), alone or in combination with the other treatments described above. We observed that AICAR reduced protein degradation by 43%, in agreement with previous reports ([10], [11]). The combined action of AICAR plus lactacystin made an extra 18% reduction in protein degradation respect to the levels obtained with lactacystin alone, indicating that AICAR was able to inhibit protein degradation by a non-proteasomal mechanism. In the same way, the combined action of AICAR plus NH_4/Leup made an extra 7% reduction in

protein degradation respect to the levels obtained with NH_4/Leup alone, indicating that AICAR was able to inhibit protein degradation by a non-lysosomal mechanism.

2) The proteasomal-independent inhibitory properties of AICAR occur by an AMPK-independent mechanism.

As expected, AICAR treatment induced the phosphorylation of AMPK α at the Thr172 residue, a sign of AMPK activation (Fig. 2A, lanes corresponding to uninfected cells). In order to assess whether the inhibitory properties of AICAR on protein degradation were related to activation of AMPK, we infected fibroblasts with an adenovirus expressing a dominant negative form of AMPK (Ad-DN-AMPK, AMPK α 1 D157A; kindly provided by Dr. Pascal Ferre and Dr. F. Foufelle) to prevent AMPK activity, or with an adenovirus expressing GFP (Ad-GFP), as a control. As it can be observed in Fig. 2A, only the infection of fibroblasts with Ad-DN-AMPK adenovirus, prevented the AICAR-induced phosphorylation of endogenous AMPK α at Thr172.

We then analyzed protein degradation in Ad-GFP and AD-DN-AMPK infected cells that were treated with AICAR, lactacystin or a combination of these two compounds. Interestingly, fibroblasts infected with Ad-DN-AMPK adenovirus showed an increase in protein degradation, suggesting a positive role of AMPK in preventing protein degradation (Fig. 2B). However, in Ad-DN-AMPK infected fibroblasts, AICAR was still able to reduce protein degradation, what indicated that the action of AICAR was performed by an AMPK-independent mechanism. To confirm these results, we infected fibroblasts with adenovirus expressing a constitutively active form of AMPK (Ad-CA-AMPK; AMPK α 1₁₋₃₁₂ T172D; kindly provided by Dr. Pascal Ferre and Dr. F. Foufelle). As it is shown in Fig. 2B, treatment of these cells with AICAR reduced protein degradation to a similar extent as in cells infected with Ad-GFP adenovirus. The

fact that we did not observe differences in protein degradation when fibroblasts were infected with the adenovirus expressing the constitutively active form of AMPK (Ad-CA-AMPK) may suggest that the basal activity of AMPK is sufficient to regulate protein degradation. At any rate, these results indicated that AICAR had AMPK-independent inhibitory properties on protein degradation.

When Ad-DN-AMPK infected cells were treated with lactacystin, protein degradation was reduced to similar levels to those obtained in Ad-GFP infected cells also treated with lactacystin. This result may suggest that the increase in protein degradation observed in Ad-DN-AMPK infected cells was due to increased proteasomal activity, what could be an indication of a positive role of AMPK in preventing proteasomal-dependent protein degradation. If Ad-DN-AMPK infected cells were treated with a combination of AICAR plus lactacystin, a further reduction in protein degradation was observed, confirming that AICAR could prevent protein degradation by an AMPK- and proteasomal-independent mechanism.

3) AICAR treatment prevents lysosomal-dependent protein degradation by decreasing the amount of autophagic vacuoles.

Since the results presented so far suggested that AICAR had proteasomal-independent inhibitory properties, we analyzed the lysosomal pathway in cells treated or not with AICAR. We observed that AICAR treatment decreased the number of autophagic vacuoles (Fig. 3A). Interestingly, this effect was also observed in cells infected with Ad-DN-AMPK adenovirus (Fig. 3A). This result indicated that the reduction in the amount of autophagic vacuoles produced by AICAR was AMPK-independent.

The inhibitory properties of AICAR on lysosomal-dependent protein degradation were confirmed by analysis of the post-translational modification of LC3-I, a cytosolic component of the autophagic pathway [18]. Upon autophagy stimulation, lipid conjugation of LC3-I leads to the formation of its autophagic-vesicle-associated form (LC3-II), which has an increased electrophoretic mobility on gels compared with LC3-I. The formation of the LC3-II conjugate is used as a marker of autophagy, since it decreases under conditions of low autophagic activity ([12], [19]) (see lanes of Fig. 3B corresponding to fibroblasts grown in Krebs-Henseleit medium with insulin and amino acids, IAa). When fibroblasts were grown in Krebs-Henseleit medium in the presence of AICAR, we observed a decrease in the formation of the LC3-II conjugate, what was an indication of inhibition of autophagy (Fig. 3B).

Autophagy is a highly regulated process that is activated under starvation conditions. On the contrary, it is inhibited when nutrient supplies are present ([6], [20], [21]). Insulin also inhibits autophagy by a mechanism involving activation of class I PI3-kinase, what leads to the subsequent activation of the Akt/PKB protein kinase by phosphorylation of Thr308 and Ser473, which eventually results in mTOR activation (a major negative regulator of autophagy) ([6], [20], [21]). Since, it has been recently suggested that AICAR could affect PI3-kinase activity [11], we checked whether AICAR treatment affected the phosphorylation status of Akt/PKB at Ser473, as a possible mechanism to explain the observed reduction in autophagy. However, AICAR treatment did not induced phosphorylation of Akt/PKB neither at Ser473 (Fig. 3C) nor at Thr308 (not shown). We repeated the analysis in cells that had been infected with the Ad-GFP adenovirus. In agreement with previous reports [22], adenovirus infection activates class I PI3-kinase leading to the phosphorylation of Akt/PKB at Ser473. However, AICAR treatment did not modify the amount of the phosphorylated form

(Fig. 3C). Therefore the inhibitory effects of AICAR on autophagy can not be explained by a stimulatory action on class I PI3-kinase activity.

It has been described that two distinct classes of PI3-kinases control autophagy in opposite directions. Whereas the action of class I PI3-kinase is inhibitory, class III PI3-kinase activates autophagy [23]. The activity of class III PI3-kinase depends on the formation of a multiprotein complex among class III PI3-kinase, beclin-1 and additional components (see [21], for review). In order to explain the inhibitory effects of AICAR on autophagy, we checked whether AICAR affected the formation of the complex between class III PI3-kinase and beclin-1. As shown in Fig. 3D, we observed that under conditions of AICAR treatment, beclin-1 could not co-immunoprecipitate with class III PI3-kinase, suggesting that the formation of the complex was defective under these conditions. Therefore, the effect of AICAR on decreasing lysosomal-dependent protein degradation could be explained by an impairment of class III PI3-kinase function.

4) AMPK downregulates proteasomal-dependent protein degradation.

As we have described in Fig. 1, in addition to its lysosomal-dependent inhibitory properties, AICAR was also able to prevent protein degradation by a possible proteasomal-dependent mechanism. To analyze whether these effects of AICAR were dependent on AMPK activity, we analyzed the activity of the proteasome in wild type and AMPK α subunit double knockout ($\alpha 1^{-/-}$, $\alpha 2^{-/-}$) mouse embryonic fibroblasts (MEFs) under conditions of AMPK activation (treatment with either AICAR [9] or metformin [24]) (Fig. 4). We observed that in wild type MEFs, both AICAR and metformin treatments reduced the activity of the proteasome by around 20%. This inhibition was significant, although much lower than the one observed by the treatment of the same cells with lactacystin (97% inhibition). However, in the double knockout

$\alpha 1^{-/-}$, $\alpha 2^{-/-}$ MEFs, neither AICAR nor metformin treatments decreased significantly the activity of the proteasome, which was however still sensitive to the action of lactacystin. These results indicated that activation of AMPK by either AICAR or metformin decreased proteasomal-dependent protein degradation.

DISCUSSION

In this work, we have examined the possible role of AMPK in regulating intracellular protein degradation. It has been reported that AMPK activation stimulates autophagy ([11], [12], [13]). The mechanism by which AMPK activates autophagy relies on the phosphorylation of the TSC1/TSC2 complex, a negative regulator of mTOR kinase; AMPK phosphorylates TSC2 leading to its activation [25], what in turn leads to the inhibition, *via* Rheb, of mTOR kinase, a major negative regulator of autophagy [21]. However, we have found that AICAR, a known AMPK activator, counteracts and reverses the effect of AMPK activation on autophagy by an AMPK-independent mechanism. AICAR impairs the formation of a functional complex of beclin-1 with class III PI3-kinase, a major positive regulator of autophagy. In this way, the overall effect of AICAR is inhibition of autophagy. This AMPK-independent effect of AICAR is similar to the one described for 3-methyladenine, LY294002 and wortmannin, three inhibitors of autophagy [26]. These compounds inhibit both class I and class III PI3-kinases and, although by inhibiting class I PI3-kinase they should stimulate autophagy because this should lead to an inhibition of the mTOR pathway, their net effect is inhibition of autophagy since they inhibit the formation of phosphatidylinositol-3-phosphate by class III PI3-kinase, which is needed for autophagy [21].

AMPK also affects proteasomal degradation, the other major intracellular protein degradation process. We have found that AMPK activation, either by AICAR or by an alternative activator such as metformin, leads to an inhibition of proteasomal function. This effect is dependent on AMPK activity since it is absent in cells that do not contain the two AMPK catalytic subunits. Although we have found that this effect is not severe when compared to the effects of proteasome inhibitors such as lactacystin, AMPK could be engaged in the fine tuning of proteasome activity under specific conditions.

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FIGURE LEGENDS

Fig. 1: AICAR affects protein degradation by proteasome-independent and lysosome-independent mechanisms. Exponentially growing human fibroblasts were metabolically labeled with [^3H]valine as described in Materials and Methods. Cells were then switched to Krebs-Henseleit (KH) medium for 4h with the following additions (final concentration): 0.5 mM AICAR, 20 μM lactacystin (Lact), 20 mM NH_4Cl plus 100 μM leupeptin (NH_4/Leup). The results are the mean from four separate experiments with duplicate samples and are expressed as percentage of the KH values (2.49% protein degraded per 1.5h). Bars indicate standard deviation and stars indicate differences which were found to be statistically significant at * $p < 0.05$ and ** $p < 0.01$ (n: 4).

Fig. 2: Protein degradation under conditions of AMPK inactivation. A) Human fibroblasts were infected with adenovirus expressing a dominant negative form of AMPK (Ad-DN-AMPK) or with adenovirus expressing GFP (Ad-GFP) as control, or left uninfected. Twenty four hours later, the cells were incubated in Krebs-Henseleit medium in the absence or in the presence of AICAR (A, 0.5 mM) or lactacystin (L, 20 μM) for 4h. Cell lysates were analyzed by western blotting using anti-phosphoT172-AMPK (upper panel), anti-AMPK α (upper middle panel), anti-GFP (lower middle panel) and anti-actin (lower panel) antibodies. B) Human fibroblasts were labeled with [^3H]valine for 48 h, and then they were infected with adenovirus encoding either a dominant negative (DN-AMPK) or a constitutively active (CA-AMPK) form of AMPK or GFP (GFP), and after 24h they were incubated in Krebs-Henseleit medium with the indicated additions as described in the legend to Fig. 1. Protein degradation was measured also as described in that figure. Results are the mean from four separate experiments with duplicated samples and are expressed as percentage of the Ad-GFP

infected cell values (1.44% protein degradation per 1.5h). Bars indicate standard deviation and stars indicate differences which were found to be statistically significant at * $p < 0.05$ and ** $p < 0.01$ (n: 4).

Fig. 3: AICAR inhibits autophagy by an AMPK-independent mechanism. A) Human fibroblasts infected with adenovirus encoding GFP (Ad-GFP) or encoding a dominant negative form of AMPK (Ad-DN-AMPK) and incubated for 4h in Krebs-Henseleit medium without or with the addition of AICAR (0.5 mM) were analyzed by electron microscopic morphometry. Bars indicate standard deviation and stars indicate differences which were found to be statistically significant at * $p < 0.05$ and *** $p < 0.001$ (n: 4). B) Cell extracts from human fibroblasts grown in Krebs-Henseleit without (KH) or with insulin and amino acids (IAa) medium treated or not with 0.5 mM AICAR for 4 hours were analyzed by immunoblotting using anti-LC3 and anti-actin antibodies. AICAR decreased the ratios of LC3-II to actin from 1.14 to 0.70 and from 0.44 to 0.33 in KH medium without or with IAa, respectively. C) Human fibroblasts were infected or not with adenovirus encoding GFP (Ad-GFP). Twenty four hours after the infection, fibroblasts were incubated for 4h in Krebs-Henseleit medium with or without the addition of AICAR (0.5 mM). Cell lysates were analyzed by western blotting using anti-phosphoS473-Akt (upper panel), anti-Akt (middle panel) and anti-phosphoT172-AMPK (lower panel) antibodies. D) Human fibroblasts were incubated for 4h in Krebs-Henseleit medium with or without the addition of AICAR (0.5 mM). Crude extracts (450 μ g) were immunoprecipitated with anti-class III PI3-kinase antibody and the immunoprecipitates analyzed by western blotting using anti-beclin-1 antibody. Crude extracts (CE, 75 μ g) were also analyzed using the same anti-class III PI3-kinase antibodies.

Fig. 4: AMPK downregulates proteasomal-dependent protein degradation. Proteasomal activity was measured in wild type and AMPK α subunit double knockout ($\alpha 1^{-/-}$, $\alpha 2^{-/-}$) MEFs incubated for 4h in Krebs-Henseleit medium with or without the addition of 0.5 mM AICAR, 2 mM metformin (Metf) or 20 μ M lactacystin (Lact). Values are the mean of at least six independent experiments. Bars indicate standard deviation and stars indicate differences which were found to be statistically significant at ** $p < 0.01$ and *** $p < 0.001$ (n: 6).

Fig. 1

Fig. 2

Fig. 3

Fig. 4