AMP-ACTIVATED PROTEIN KINASE AND THE REGULATION OF AUTOPHAGIC PROTEOLYSIS

Daniel Meley*, Chantal Bauvy*, Judith H.P.M. Houben-Weerts‡, Peter F. Dubbelhuis‡, Mariette T.J. Helmond‡, Patrice Codogno* and Alfred J. Meijer¶¶

*INSERM U756, Faculté de Pharmacie, Université Paris-Sud 11, 92296 Châtenay-Malabry, France
†Department of Medical Biochemistry, Academic Medical Center, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands.

Running title: Control of macroautophagy by AMP-activated protein kinase

¶¶To whom correspondence should be addressed: Department of Medical Biochemistry, Academic Medical Center, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands. Tel. 31-20-5665159; Fax 31-20-6915519; E-mail: a.j.meijer@amc.uva.nl

 Interruption of mTOR-dependent signalling by rapamycin is known to stimulate autophagy, both in mammalian cells and in yeast. Because activation of AMPK also inhibits mTOR-dependent signalling one would expect stimulation of autophagy by AMPK activation. According to the literature, this is true for yeast but, unexpectedly, not for mammalian cells on the basis of the use of AICAR, a pharmacological activator of AMPK. In the present study, carried out with hepatocytes, HT-29 cells, and HeLa cells, we have reexamined the possible role of AMPK in the control of mammalian autophagy. Inhibition of AMPK activity by compound C or by transfection with a dominant negative form of AMPK almost completely inhibited autophagy. These results suggest that the inhibition of autophagy by AICAR is not related to its ability to activate AMPK. We conclude that in mammalian cells, as in yeast, AMPK is required for autophagy.

During macroautophagy (hereafter referred to as autophagy), a small part of the cytoplasm is sequestered by a double isolation membrane, presumably derived from the endoplasmic reticulum, to form an autophagosome. This autophagosome then fuses with a lysosome and the sequestered macromolecular material, including proteins, is degraded. The process is activated by various stress situations, including nutrient depletion. Under these conditions, autophagy provides the constituents required to maintain the metabolism essential for survival (1, 2). An example is that of the mammalian liver in which autophagy is accelerated during fasting to provide amino acids for gluconeogenesis, in order to meet the energy requirements of the brain and of erythrocytes.

In many cell types, including liver cells, autophagy is inhibited by amino acids, in synergy with insulin, and this inhibition is mediated, at least in part, by mTOR-dependent signalling (1). Depending on the cell type and the conditions, other signalling pathways, such as the ras/raf/MAPK signalling pathway, may also participate in amino acid control of autophagy (1). In addition, autophagy is controlled by phosphatidylinositol phospholipids. The process is inhibited by PtdIns(3,4,5,)P3, the product of PI3K class I, a lipid kinase located upstream of mTOR in the insulin-signalling pathway. By contrast, PI(3)P, the product of PI3K class III, is essential for autophagy (1, 3, 4). This requirement for PI(3)P explains why PI3K inhibitors are also autophagy inhibitors. Indeed, the classical autophagy inhibitor 3-methyladenine (5) turned out to be a PI3K inhibitor (1).

After the original observation in 1995 that amino acids can stimulate mTOR-dependent signalling (6), it is now generally accepted that the mTOR pathway acts as a sensor of amino acids (7). A few years ago we, and others, discovered that mTOR can also sense changes in the cellular energy state via AMP-activated protein kinase (AMPK)1. Activation of this protein kinase inhibits mTOR-dependent signalling and inhibits protein synthesis (8), which is consistent with AMPK’s function of switching off ATP-dependent processes (9).

Inhibition of mTOR by AMPK, like that caused by addition of rapamycin (1, 2), may be expected to increase autophagy. However, in the literature there is controversy on this issue. In yeast, activation of AMPK stimulates autophagy (10). By contrast, activation of...
AMPK by addition of the cell-permeable nucleotide analogue AICArbside (AICAR) in hepatocytes strongly inhibits autophagy (11). In the present study, using different mammalian cell types, we have examined the possible role of AMPK in the control of autophagy in more detail. Our data indicate that AMPK, like in yeast, is required for autophagy.

MATERIALS AND METHODS

Materials - Cell culture products were from Gibco (Carlsbad, USA). Insulin, rapamycin, AICAR (AICArbside, imidazole-4-carboxamide 1-β-ribofuranoside), the chemicals for enhanced chemiluminescence (ECL), BCA kits, Ponceau red, metformin and protease inhibitors cocktail were from Sigma (St. Louis, MO, USA). Compound C was a gift of Merck Sharp & Dohme BV (Haarlem, The Netherlands). The FuGENE 6™ transfection kit was from Roche (Basel, Switzerland) and the Lipofectamin™ 2000 was from Invitrogen (Carlsbad, USA). L-[U-14C]-valine was from PerkinElmer Life Sciences (Boston, USA) and L-valine was from Sigma (St. Louis, MO, USA). L-[U-14C]-valine was from Sigma (St. Louis, MO, USA). L-[U-14C]-valine was from Sigma (St. Louis, MO, USA). L-[U-14C]-valine was from Sigma (St. Louis, MO, USA). L-[U-14C]-valine was from Sigma (St. Louis, MO, USA).

Hepatocytes - Hepatocytes were isolated from male Wistar rats (250-300 g) starved for 16 to 20 h by collagenase perfusion (6). Hepatocytes (5 mg dry weight/ml) were incubated for the indicated times at 37°C in minimal medium (Krebs–Henseleit bicarbonate buffer plus 10 mM Na+–Hepes, pH 7.4, and 20 mM glucose) plus the components as indicated in the legends. The final incubation volume was 2 ml. The gas atmosphere was O2/CO2 (19:1, v/v).

At the end of the incubations, hepatocytes were collected for gel analysis by centrifugation in 5 volumes of an ice-cold solution of 150 mM NaCl plus 10 mM Na–Hepes (pH 7.4) for 5 s in an Eppendorf centrifuge. For the SDS–PAGE procedures, the pellet was lysed by addition of Laemmli sample buffer and subsequently incubated at 95°C for 5 min.

For determination of ATP, lactate and of amino acids, an aliquot of the incubated cell suspension was acidified with HClO4 (final concentration, 3%, m/v). After removal of the precipitated protein by centrifugation in a microcentrifuge (1 min; 10,000g), the clear supernatant was neutralized to pH 7 with a small volume of a mixture of 2 M KOH plus 0.3 M Mops.

Cell culture - HT-29 human colon cancer cells and HeLa cells were maintained in DMEM 4.5 g/L glucose supplemented with 10% foetal bovine serum and 1% penicillin/streptomycin solution. Medium was replaced three times per week, and cells were passaged at confluence. The cells were grown in a humidified atmosphere of 10% CO2–90% air at 37°C. Cells were plated and grown to 50-80% confluency before treatment for different times.
with vehicle or adequate concentrations of amino acids (4X), rapamycin (100 nM), metformin (1-10 mM), 3-methyladenine (3-MA) (10 mM), IL-13 (30 ng/ml) and AICAR (0.1-1 mM).

**Determination of ATP, lactate, AICAR and ZMP** - ATP was determined fluorimetrically with NADP+, glucose, hexokinase and glucose 6-phosphate dehydrogenase (13). Lactate was measured spectrophotometrically with NAD+ and lactate dehydrogenase (13). AICAR and ZMP was measured by HPLC as described by Samari and Seglen (11).

**Transfection** - The expression constructs pEGFP-C1-LKB1wt, pcDNA3-FLAG-STRAD, pcDNA3-myc-AMPKCA, pcDNA3-myc-AMPKD and control vectors were introduced into HT-29 cells and HeLa cells using Lipofectamin™ 2000 and the FuGENE 6™ Reagent transfection kit, respectively. Transfected cells were cultured in complete medium for 48 h before use for the different experiments. Expression levels of each construct were determined by SDS-PAGE analysis using the relevant Tag antibody.

**Immunoblotting** - After SDS-PAGE resolution, the proteins were transferred on nitrocellulose membrane. The membranes were blocked with 5% nonfat dry milk in TBST [10 mM Tris-HCl (pH=8); 100 mM NaCl; 0.1% Tween 20] for 1 h at room temperature and then incubated with appropriate primary antibody overnight at 4°C (diluted in TBST–5% BSA), followed by incubation with horseradish peroxidase-conjugated secondary antibody at 1:5000 dilution in TBST-5% BSA. All other total antibodies were used at 1:1000 dilution in TBST-5% BSA, and anti-phospho antibodies were diluted at 1:1000 in TBST-1% BSA. The anti-c-myc were used at 1:200 dilution in TBST-5% BSA. The anti-FLAG was used at 1:1000 dilution.

To quantify the different spots of immunoblotting, we used the freeware Scion Imaging (www.scioncorp.com).

**Autophagic parameters**

**Amino acid analysis** - Amino acids were analysed with HPLC exactly as described by (14). Of the branched-chain amino acids, the valine peak in the amino acid spectrum was contaminated with a compound of unknown origin, and was therefore not used.

**[^14C]Chloroquine accumulation** - Accumulation of the divalent weak base chloroquine, which monitors changes in the pH of intracellular acidic compartments, mainly lysosomes, was measured exactly as described elsewhere (15). In these experiments, the hepatocyte concentration was 1 mgdw/ml, the concentration of chloroquine was <1 µM and the amount of radioactivity was 0.025 µCi/ml of incubation medium.

**Measurement of the degradation of long-lived proteins** - Proteolysis was determined as described previously (16). Briefly, cells were incubated for 24 h at 37°C with 0.05 µCi/ml of L-[U-^14C]valine. Unincorporated radiisotope was removed by three rinses with phosphate-buffered saline (PBS). Cells were incubated in complete medium, supplemented with 10 mM cold valine throughout the pre-chase period. After 1 h of pre-chase, the medium was removed by three rinses with PBS containing 0.9 mM CaCl₂ and 0.5 mM MgCl₂ (PBS+) and a complete mixture of amino acids in which each amino acid was present at 4 times its concentration in the portal vein of a 24-h-fasted rat (for composition, see (6)), rapamycin (100 nM), metformin (2 mM), AICAR (250 µM), 3-MA (10 mM) or IL-13 (30 ng/ml) in nutrient-free medium (without amino acids and in the absence of foetal calf serum), HBSS or Hanks balanced saline solution, supplemented with 0.1% bovine serum albumin and 10 mM cold valine were added at the beginning of the chase period. During the pre-chase, the short-lived proteins were being degraded. The chase continued for 4 h. Cells and radiolabelled proteins from the 4 h chase medium were precipitated in trichloroacetic acid at a final concentration of 10% (w/v) at 4 °C overnight. After centrifugation, pellets were dissolved in 0.2 N NaOH. Radioactivity was determined by liquid scintillation counting. Protein degradation was calculated by dividing the acid-soluble radioactivity recovered from both cells and medium by the radioactivity contained in the precipitated proteins from both cells and medium.

**Monodansylcadaverine staining** -
Monodansylcadaverine (MDC) staining was performed as previously described (17). Briefly, cells were seeded on microscope cover glasses. After 3 days, cells were treated for 2 h in appropriate mediums, and during the last 10 min 0.1 µM MDC was added. Cells were then washed by three rinses with PBS+ and fixed with a solution of 3% paraformaldehyde for 20 min. The microscope cover glasses were washed again by three rinses with PBS+, and put on microscope slides with Mowiol. The slides were incubated overnight at 4°C and observed in the epifluorescence microscope. Only cells expressing GFP-LKB1 were counted for MDC staining.

**Statistics** - Data were summarized as mean ± S.E.. Statistical significance was determined using Student’s t-test (p<0.05).

**RESULTS**

*Effect of pharmacological modulators of AMPK activity on autophagic proteolysis in isolated rat hepatocytes*

When hepatocytes are incubated in the absence of amino acids, autophagic proteolytic flux is maximal. As previously reported by (11), addition of the AMPK activator AICAR under these conditions strongly inhibited autophagy, as indicated by the large reduction in the appearance of the branched-chain amino acids isoleucine and leucine (which are not further catabolised in rat liver) (Fig. 1A,B). This was also accompanied by reductions in the major gluconeogenic amino acids aspartate, glutamate, glutamine and alanine (Fig. 1C-F). It should be stressed that these experiments were carried out in the presence of a low concentration of cycloheximide (10 µM) to prevent reincorporation of the proteolytically formed amino acids into protein. The inhibition of proteolysis by AICAR was similar to that observed with the autophagy inhibitor 3-methyladenine and also to that observed with the acidotropic agent methylamine which inhibits lysosomal function by raising the lysosomal pH (Fig. 1A,B).

Administration of the antidiabetic agent metformin is another way to stimulate AMPK activity (18). The effects of AICAR and metformin on AMPK phosphorylation are compared in Fig. 2. In the presence of AICAR (250 µM), phosphorylation of AMPK was rapid but decreased after 40 min, presumably because continuous intracellular accumulation of ZMP results in ZMP levels high enough to inhibit AMPK (19). By contrast, in the presence of 2 mM metformin, AMPK phosphorylation was initially slow (at 40 min it was similar to that seen with AICAR at 20 min) and increased with time to a maximum at 80 min. Even though 2 mM metformin was more potent than AICAR in stimulating AMPK phosphorylation, the ability of metformin to inhibit autophagic production of the branched-chain amino acids was less than that observed with AICAR (Fig. 1A,B). Furthermore, production of glutamine, glutamate and aspartate was not significantly affected by metformin while that of alanine was even substantially increased (Fig. 1C-F), presumably because glycolytic flux increased in the presence of metformin (see below).

While AICAR and metformin are AMPK activators, we also wanted to study the effect of inhibition of AMPK activity on autophagy. Compound C has been reported as a specific inhibitor of AMPK (18), and we examined its efficacy in this regard.

Metformin-stimulated phosphorylation of AMPK was reversed by compound C (Fig. 3). Compound C did not inhibit AICAR-stimulated AMPK phosphorylation unless amino acids were also present (Fig.4) (see Discussion). The same was true for acetylCoA carboxylase (ACC), which is a substrate for AMPK (Fig. 4). It was shown previously that compound C, in addition to its action as an inhibitor of AMPK (18), and we examined its efficacy in this regard.

Metformin-stimulated phosphorylation of AMPK was reversed by compound C (Fig. 3). Compound C did not inhibit AICAR-stimulated AMPK phosphorylation unless amino acids were also present (Fig.4) (see Discussion). The same was true for acetylCoA carboxylase (ACC), which is a substrate for AMPK (Fig. 4). It was shown previously that compound C, in addition to its action as an inhibitor of AMPK (18), and we examined its efficacy in this regard.

Metformin-stimulated phosphorylation of AMPK was reversed by compound C (Fig. 3). Compound C did not inhibit AICAR-stimulated AMPK phosphorylation unless amino acids were also present (Fig.4) (see Discussion). The same was true for acetylCoA carboxylase (ACC), which is a substrate for AMPK (Fig. 4). It was shown previously that compound C, in addition to its action as an inhibitor of AMPK (18), and we examined its efficacy in this regard.

Metformin-stimulated phosphorylation of AMPK was reversed by compound C (Fig. 3). Compound C did not inhibit AICAR-stimulated AMPK phosphorylation unless amino acids were also present (Fig.4) (see Discussion). The same was true for acetylCoA carboxylase (ACC), which is a substrate for AMPK (Fig. 4). It was shown previously that compound C, in addition to its action as an inhibitor of AMPK (18), and we examined its efficacy in this regard.

Metformin-stimulated phosphorylation of AMPK was reversed by compound C (Fig. 3). Compound C did not inhibit AICAR-stimulated AMPK phosphorylation unless amino acids were also present (Fig.4) (see Discussion). The same was true for acetylCoA carboxylase (ACC), which is a substrate for AMPK (Fig. 4). It was shown previously that compound C, in addition to its action as an inhibitor of AMPK (18), and we examined its efficacy in this regard.

Metformin-stimulated phosphorylation of AMPK was reversed by compound C (Fig. 3). Compound C did not inhibit AICAR-stimulated AMPK phosphorylation unless amino acids were also present (Fig.4) (see Discussion). The same was true for acetylCoA carboxylase (ACC), which is a substrate for AMPK (Fig. 4). It was shown previously that compound C, in addition to its action as an inhibitor of AMPK (18), and we examined its efficacy in this regard.

Metformin-stimulated phosphorylation of AMPK was reversed by compound C (Fig. 3). Compound C did not inhibit AICAR-stimulated AMPK phosphorylation unless amino acids were also present (Fig.4) (see Discussion). The same was true for acetylCoA carboxylase (ACC), which is a substrate for AMPK (Fig. 4). It was shown previously that compound C, in addition to its action as an inhibitor of AMPK (18), and we examined its efficacy in this regard.

Metformin-stimulated phosphorylation of AMPK was reversed by compound C (Fig. 3). Compound C did not inhibit AICAR-stimulated AMPK phosphorylation unless amino acids were also present (Fig.4) (see Discussion). The same was true for acetylCoA carboxylase (ACC), which is a substrate for AMPK (Fig. 4). It was shown previously that compound C, in addition to its action as an inhibitor of AMPK (18), and we examined its efficacy in this regard.

Metformin-stimulated phosphorylation of AMPK was reversed by compound C (Fig. 3). Compound C did not inhibit AICAR-stimulated AMPK phosphorylation unless amino acids were also present (Fig.4) (see Discussion). The same was true for acetylCoA carboxylase (ACC), which is a substrate for AMPK (Fig. 4). It was shown previously that compound C, in addition to its action as an inhibitor of AMPK (18), and we examined its efficacy in this regard.

Metformin-stimulated phosphorylation of AMPK was reversed by compound C (Fig. 3). Compound C did not inhibit AICAR-stimulated AMPK phosphorylation unless amino acids were also present (Fig.4) (see Discussion). The same was true for acetylCoA carboxylase (ACC), which is a substrate for AMPK (Fig. 4). It was shown previously that compound C, in addition to its action as an inhibitor of AMPK (18), and we examined its efficacy in this regard.

Metformin-stimulated phosphorylation of AMPK was reversed by compound C (Fig. 3). Compound C did not inhibit AICAR-stimulated AMPK phosphorylation unless amino acids were also present (Fig.4) (see Discussion). The same was true for acetylCoA carboxylase (ACC), which is a substrate for AMPK (Fig. 4). It was shown previously that compound C, in addition to its action as an inhibitor of AMPK (18), and we examined its efficacy in this regard.

Metformin-stimulated phosphorylation of AMPK was reversed by compound C (Fig. 3). Compound C did not inhibit AICAR-stimulated AMPK phosphorylation unless amino acids were also present (Fig.4) (see Discussion). The same was true for acetylCoA carboxylase (ACC), which is a substrate for AMPK (Fig. 4). It was shown previously that compound C, in addition to its action as an inhibitor of AMPK (18), and we examined its efficacy in this regard.
AICAR inhibited amino acid-stimulated, rapamycin-sensitive, phosphorylation of p70S6 Kinase, in agreement with our earlier observations (21). This effect was largely prevented by 40 μM compound C (Fig. 4). AICAR alone slightly stimulated p70S6 kinase phosphorylation (Fig. 4), in agreement with data of Moller et al. (22) who showed that this phosphorylation was rapamycin-insensitive.

Having thus established that compound C can inhibit AMPK activity in intact hepatocytes, whether by a direct effect on the enzyme or indirectly by competing with AICAR for transport into the cells (20), we tested its effect on autophagy. If AMPK activation inhibits autophagy as suggested (11), one would expect compound C to be able to reverse the inhibition of autophagy by metformin. Unexpectedly, however, compound C inhibited autophagic proteolysis when added alone and the effect was not additive with either that of metformin, AICAR or 3-methyladenine (Fig. 1A and B).

In order to rule out the possibility that compound C inhibited the lysosomal proton pump, we tested its effect on the intracellular accumulation of [14C]chloroquine, a divalent weak base which when present at low concentrations greatly accumulates in acidic intracellular compartments, mainly lysosomes (15). As a control, the effect of 5 mM methylamine was also tested. Chloroquine accumulation was not affected by compound C (Fig. 5), but methylamine greatly reduced it, as expected. AICAR significantly decreased chloroquine accumulation although not to the same extent as methylamine, whereas metformin had no significant effect.

We also examined whether variations in glycolysis were in some way associated with the observed changes in autophagic proteolysis, but this was not the case. Omission of glucose did not affect autophagic proteolysis (Fig. 1A,B) and lactate was not formed under these conditions (Fig. 1G). ATP levels and AMPK phosphorylation were not affected by glucose depletion (not shown), presumably because mitochondrial oxidation of endogenous fatty acids provided sufficient energy. Metformin stimulated, while both AICAR and 3-methyladenine strongly inhibited production of lactate; compound C, on the other hand, had no effect on lactate formation (Fig. 1G). The effect of metformin on the production of lactate is consistent with the ability of this compound to act as a weak inhibitor of the mitochondrial respiratory chain (23, 24). Indeed, we observed a decrease in intracellular ATP levels from 11.2 ± 0.9 to 6.2 ± 0.2 μmol/g dry weight of cells after 90 min of incubation in the presence of 2 mM metformin (n=5; p<0.05) (data not shown). The inhibition of glycolysis by AICAR and 3-methyladenine is in agreement with previous observations in hepatocytes (25, 26).

In the course of our experiments, we noted that AICAR inhibited insulin-stimulated phosphorylation of protein kinase B, which was prevented by amino acids but not by compound C (Fig. 4). These data indicate that, in addition to activating AMPK, AICAR may have other effects (see Discussion).

**Inhibition of AMPK activity blocks autophagic proteolysis in human cell lines**

We have previously shown that autophagic proteolysis is stimulated when the human colon carcinoma HT-29 cells are incubated in nutrient-free medium (4). Pilot experiments showed that metformin and AICAR were able to activate AMPK in HT-29 cells, as determined by its phosphorylation at position Thr172 and phosphorylation of its substrate ACC at position Ser79, with a maximal effect at 250 μM AICAR and 2 mM metformin (Fig. 6A and data not shown). Whatever the concentration used, cell viability was greater than 95% under the experimental conditions used in this study. Following on with these results, we next investigated the effect of AICAR and metformin on the degradation of long-lived [14C]-valine-labelled proteins. Both compounds inhibited the degradation of [14C]-valine-labelled proteins in nutrient-free medium to the same extent (Fig. 6B). However, only a partial inhibition of proteolysis was observed when compared to 3-MA, amino acids or interleukin 13, known inhibitors of autophagic proteolysis in HT-29 cells (4) (Fig. 6B). In order to correlate these findings with the state of AMPK activation, HT-29 cells were transfected with a constitutively-active form of AMPK (AMPKCA). As previously shown (27), AMPK activity was increased, as determined here by the phosphorylation of the AMPK substrate ACC, in cells expressing AMPKCA (Fig. 7A). Next, we have analyzed the rate of degradation of long-lived [14C]-valine-labelled
proteins in cells expressing AMPKCA. As shown in Fig. 7B, the rate of degradation of long-lived \[^{14}C\] -valine-labelled proteins and its sensitivity to autophagy inhibitors were similar to that observed in untransfected HT-29 cells (Fig. 6B) and in cells transfected with an empty vector (data not shown) when incubated in nutrient-free medium. From these findings we reasoned that the inhibition of AMPK activity should inhibit the rate of autophagic proteolysis in cells incubated in nutrient-free medium. For this purpose, we used two approaches. In a first approach, cells were transfected with the cDNA encoding a dominant-negative form of AMPK (AMPKDN). In a second approach, cells were treated with compound C. According to previous results (27), activation of AMPK activity by AICAR was impaired in cells expressing AMPKDN (Fig. 7A). Moreover starvation-induced proteolysis was inhibited in these cells when compared to untransfected cells (Fig. 6B), to cells expressing AMPKCA (Fig. 7B) and to cells transfected with an empty vector (data not shown). In a second set of experiments we have analyzed the rate of degradation of long-lived \[^{14}C\]-valine-labelled proteins in the presence of compound C (40 \(\mu\)M). Prior to this, we verified the inhibitory effect of compound C on AMPK activity in HT-29 cells. For this purpose, HT-29 cells were cultured in the absence of glucose, a condition known to activate AMPK and to favor the phosphorylation of its substrate ACC (Fig. 6A). In the presence of compound C, ACC phosphorylation was inhibited by 80% in treated cells when compared to untreated cells.

In agreement with the findings in hepatocytes (cf. Fig. 1), compound C inhibited proteolysis to the same extent as autophagic proteolysis inhibitors in HT-29 cells (Fig. 6B). In addition, no additive inhibition was observed in the presence of both compound C and 3-MA (data not shown). From these findings we concluded that the inhibition of AMPK activity blocks autophagic proteolysis whereas active AMPK is required during starvation-induced autophagy. In order to exclude a cell line-dependent effect, we have next investigated the role of AMPK during autophagic proteolysis in another cell line, HeLa cells. In this cell line, which does not express the AMPK kinase LKB1 (28), AICAR is not able to stimulate AMPK unless LKB1 is expressed (Fig. 8B and ref. (29)). LKB1 is present in a complex with the regulatory proteins STRAD and MO25 (29). As the amount of STRAD is low in HeLa cells (29), we co-transfected HeLa cells with cDNAs encoding LKB1 and STRAD (Fig. 8A). In transfected cells we observed an increase in AMPK activity which was further stimulated in the presence of AICAR (Fig. 8B and ref. (29)). Next we have analyzed the rate of degradation of long-lived \[^{14}C\]-valine-labelled proteins in cells transfected with cDNAs encoding LKB1 and STRAD and in control HeLa cells (cells transfected with empty vectors). As shown in Fig. 8C, in both cell lines amino acid- and 3-MA-sensitive autophagic proteolysis was stimulated in HBSS. However, no significant difference in the rate of starvation-stimulated autophagic proteolysis was observed depending on the activity of AMPK. Although the activity of AMPK was not stimulated by AICAR, an inhibitory effect of AICAR on autophagic proteolysis was still observed (Fig. 8C). Next we analyzed the effect of inhibition of AMPK activity on the rate of proteolysis. In a first series of experiments, HeLa cells expressing LKB1 and STRAD were transfected with the cDNA encoding AMPKDN (Fig. 8A). Under these conditions, we observed a decrease in AMPK activity and no stimulatory effect of AICAR (Fig. 8B). We observed an almost complete inhibition of starvation-induced autophagic degradation of long-lived \[^{14}C\]-valine-labelled proteins in these cells (Fig. 8C). We then investigated the effect of compound C in HeLa cells and in cells expressing LKB1 and STRAD. Compound C inhibited AMPK activity in HeLa cells expressing LKB1 and STRAD and also in control HeLa cells which have a low but detectable AMPK activity (Fig. 8B). In agreement with the results obtained in hepatocytes and HT-29 cells, compound C was a very potent inhibitor of starvation-induced autophagic proteolysis in HeLa cells, independent of the amount of LKB1 and STRAD (Fig. 8C). As autophagy is a vacuolar mechanism that sequesters cytoplasmic material to deliver it to the lysosomal compartment, we have investigated the presence of autophagic vacuoles by staining with MDC, a dye that accumulates in acidic compartments including autophagic vacuoles (30, 31). As expected, MDC-positive vacuoles accumulated in HeLa cells expressing LKB1 and STRAD when incubated for 2 h in nutrient-free medium (Fig. 9). According to
previous studies (31), the number of MDC-positive vacuoles was reduced by 80-85% in cells incubated in nutrient-free medium in the presence of 3-MA or amino acids (Fig. 9). The same qualitative and quantitative observations were also made in control HeLa cells (data not shown). However, expression of AMPKDN in HeLa cells expressing LKB1 and STRAD reduced by 65-70% the number of MDC-positive vacuoles sensitive to 3-MA or amino acids in cells incubated in nutrient-free medium (Fig. 9) when compared to cells not expressing AMPKDN. These findings strongly suggest that inhibition of AMPK activity interferes with the stimulation of autophagy in nutrient-free medium.

DISCUSSION

We have been able to confirm the observation of Samari and Seglen (11) that AICAR is a very effective inhibitor of autophagy in hepatocytes. It was proposed (11) that AICAR, after its intracellular phosphorylation to ZMP, inhibits autophagy because of its ability to activate AMPK. Because autophagy is accelerated when cells have insufficient oxidisable substrate at their disposal, inhibition of autophagy by AMPK activation under these conditions would, however, be counterproductive (1).

In hepatocytes, AMPK activity did not match the inhibition of autophagy. Our experiments show that metformin, even though it strongly stimulated AMPK phosphorylation, was less potent as an autophagy inhibitor. The partial inhibition of autophagy by metformin may have been caused by the significant decline in cellular ATP levels that was observed under these conditions. Indeed, a decrease in ATP alone, in the absence of changes in AMPK activity, has previously been shown by Moller et al. (32) to inhibit autophagy. Consequently, when nutrient depletion is too excessive, with strong reduction in intracellular ATP, autophagy may become inhibited because, after all, autophagy is a complicated membrane-flow-dependent process which does require input of ATP (33).

The fact that the AMPK inhibitor compound C strongly inhibited autophagy, not only in hepatocytes but also in HT-29 cells and HeLa cells, suggested that activated AMPK, rather than inhibiting autophagy is in fact required for autophagy, a situation similar to that in yeast (10). This was supported by the experiments with HT-29 cells and HeLa cells showing that transfection of these cells with AMPKDN completely inhibited autophagy in HBSS. By contrast, transfection with AMPKCA did not affect the rate of autophagy under these conditions. We conclude from these experiments that AMPK is essential for autophagy and that, apparently, basal activity of AMPK is sufficient for autophagy.

Our findings are in line with recent data showing that activation of the tumour suppressor p53 inhibits mTOR activity through activation of AMPK, a phenomenon that is accompanied by increased autophagy (34, 35). Our data also tally with the recently demonstrated requirement of autophagy for eukaryotic elongation factor 2-kinase (eEF-2 kinase) (36), which is known to be activated by AMPK (37).

The fact that in HeLa cells, which lack LKB1, autophagy can be inhibited by compound C (Fig. 8) leads us to conclude that, apparently, AMPK can also be phosphorylated by another upstream kinase. Major candidates are CaMKK and TAK1 (38-40).

The question to be answered now is why autophagy is inhibited by AICAR. Presumably, the inhibition of autophagy by AICAR is not related to its ability to activate AMPK. One possibility is that AICAR (or rather ZMP), like 3-methyladenine, is also a PI3K inhibitor which inhibits both PI3K class I and III, the latter being essential for autophagy (4). In PI3K class III-overexpressed Chinese Hamster Ovary cells AICAR strongly inhibited PI3K Class III activity, although the effect was assumed to be due to activation of AMPK (41). The observation that insulin-induced phosphorylation of protein kinase B was antagonised by AICAR (Fig. 4), which has also been reported by others (42-44), may be explained by PI3K inhibition. In macrophages, AICAR has been directly shown to inhibit PI3K (43). In this context, it is important to note that caffeine and theophylline, derivatives of the purine xanthine, are also inhibitors of PI3K, albeit only at high concentrations (45).

There are other observations that suggest that AICAR may not be specific as an activator of AMPK but can exert other actions. Thus, AICAR was found to inhibit glycolysis in hepatocytes (Fig. 1G). This effect, which has been reported previously (26, 46), is highly
unexpected because activation of AMPK should stimulate rather than inhibit glycolysis which, after all, is an ATP-producing process. Indeed, activation of AMPK with metformin was accompanied by increased production of lactate (Fig. 1G).

Another effect of AICAR, not related to AMPK activation because metformin had no effect, was its ability to raise the lysosomal pH (Fig. 5), perhaps due to the amine group in its chemical structure. Although acidotropic agents may not affect the rate of formation of autophagosomes (6, 47), they do inhibit the degradation of protein (and of other macromolecules) within the autophagolysosome.

Although AICAR has been widely used in studies of the role of AMPK in metabolism (9), clearly not all effects of AICAR can be ascribed to its ability to activate AMPK.

We realize that part of the evidence that AMPK is essential for autophagy relies on the use of compound C and its assumed specificity. Compound C was originally described as a specific inhibitor of AMPK activity (18) but more recent reports showed that AMPK phosphorylation itself can also be affected by compound C (cf. also Fig. 4) (48, 49). Apart from an effect of compound C on the conversion of AICAR to ZMP (20) (cf. Table 1), which can be considered as an indirect way of inhibiting AMPK activity, we have no indications that the inhibition of autophagy by compound C was due to effects other than that on AMPK. Moreover, an effect of compound C on the lysosomal pH could be ruled out (Fig. 5).

Finally, we would like to comment on the finding that not only compound C but also amino acids are able to inhibit ZMP production from AICAR, albeit by a different mechanism, so that ZMP production was almost completely blocked in the presence of both compound C and amino acids (Table 1). These observations underscore the warning (20) that variations in the activity of AMPK in the presence of AICAR are not always due to direct effects on AMPK.

Although AMPK is activated by ZMP, high concentrations of ZMP have been shown to inhibit the enzyme (19) and makes the effect of a partial decrease in intracellular ZMP on AMPK difficult to predict. The ability of compound C, in contrast to amino acids, to inhibit AICAR-induced AMPK phosphorylation in hepatocytes on its own (Fig. 4), even though the accumulation of ZMP in both cases decreased similarly (Table 1) may be ascribed to small differences in intra/extracellular distribution of ZMP. Although most of the ZMP produced from AICAR is recovered inside the hepatocytes (50), it is possible that in the presence of amino acids, when cell volume increases (51), some of the ZMP leaks to the extracellular fluid so that the total amount of ZMP in the cell suspension overestimates the actual concentration of ZMP inside the cells.

In summary, the data presented in this study confirm that the AMPK activator AICAR inhibits autophagy but do not support the conclusion that AMPK is responsible for this effect (11). Rather, our observations indicate that AMPK activation is required for autophagy, a situation similar to that in yeast (10).

REFERENCES

FOOTNOTES

We are grateful to Adrian Minty, David Carling, Marc Billaud and Hans Clevers for providing us with reagents used in this study. Thanks are also due to Merck Sharp & Dohme BV (Haarlem, The Netherlands) for compound C. This work was supported by institutional funding from the Institut National de la Santé et de la Recherche Médicale (INSERM) and from the University of Paris-Sud 11, and by a grant from the Association pour la Recherche sur le Cancer (ARC) (n°3503 to PC). DM was supported by an ARC Fellowship.

1The abbreviations used are: AICAR, AICArriboside, imidazole-4-carboxamide-1-β-ribofuranoside; ZMP, AICAR monophosphate; 3-MA, 3-methyladenine; AMPK, AMP-activated protein kinase; PBS, phosphate-buffered saline; DMEM, Dulbecco’s modified Eagle’s medium; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; mTOR, mammalian target of rapamycin; p70S6K, p70S6 kinase; 4AA, complete mixture of amino acids in which each amino acid occurs at 4x their concentration in the portal vein of a fasted rat; PBS, phosphate buffered saline; MDC, monodansylcadaverine; CC, compound C; ACC, acetylCoA carboxylase; DMSO, dimethylsulfoxide; eIF4E, eukaryotic initiation factor 4E; GFP, green fluorescent protein; gdw, gram dry weight of cells.

TABLE

Table 1. Inhibition by compound C and by amino acids of ZMP production from AICAR in hepatocytes. Hepatocytes were incubated for 100 min in the presence of 250 µM AICAR and, if present, 40 µM compound C (CC) and/or the complete mixture of amino acids (4AA). Data are the means (± S.E.) of experiments carried out with 3 different hepatocyte preparations. *p<0.05 versus control.

<table>
<thead>
<tr>
<th></th>
<th>-ΔAICAR (µmol/gdw)</th>
<th>ZMP (µmol/gdw)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>37.1 ± 1.8</td>
<td>24.2 ± 1.4</td>
</tr>
<tr>
<td>4AA</td>
<td>40.5 ± 2.1</td>
<td>10.6 ± 1.0*</td>
</tr>
<tr>
<td>CC</td>
<td>25.5 ± 1.8*</td>
<td>11.6 ± 0.8*</td>
</tr>
<tr>
<td>4AA + CC</td>
<td>27.9 ± 2.3*</td>
<td>3.6 ± 0.4*</td>
</tr>
</tbody>
</table>

FIGURE LEGENDS

Fig. 1. Effects of AICAR, metformin (Met), compound C (CC), 3-methyladenine (3MA), methylamine (Meam) and of glucose depletion on proteolysis and lactate production in hepatocytes. Hepatocytes were incubated for 90 min in the presence of 20 mM glucose (unless otherwise indicated) and cycloheximide (10 µM), in the absence or presence of 2 mM metformin, 250 µM AICAR, 5 mM 3-methyladenine, 40 µM compound C (CC) or 5 mM methylamine, or combinations thereof, as indicated. Data were obtained from experiments carried out with 3-11 different hepatocyte preparations. When no standard error is indicated, the standard error was too small to be seen. *p<0.05 versus control cells (“None”).
Fig. 2. Effect of AICAR and metformin on AMPK phosphorylation in hepatocytes. Hepatocytes were incubated in the presence of 20 mM glucose for the times indicated, in the absence or presence of 250 μM AICAR or 2 mM metformin.

Fig. 3. Effect of compound C (CC) on AMPK phosphorylation by AICAR and metformin in hepatocytes. Hepatocytes were incubated for 30 min with 20 mM glucose, in the absence or presence of 250 μM AICAR or 2 mM metformin.

Fig. 4. Effect of AICAR, compound C and rapamycin on the phosphorylation of AMPK, acetylCoA carboxylase (ACC), p70S6k and protein kinase B, in the absence and presence of amino acids in hepatocytes. Hepatocytes were incubated with 20 mM glucose for 100 min in the absence or presence of 250 μM AICAR, 100 nM rapamycin or 40 μM compound C (CC), or combinations thereof. A complete mixture of amino acids (21) (AA), if present, was added at 30 min. Insulin (10^{-7} M), if present, was added 5 min before the end of the incubation. eIF4E was used to check for the loading of the gel.

Fig. 5. Intracellular/extracellular concentration gradient of chloroquine. Hepatocytes were incubated with 20 mM glucose and \( [^{14}C] \)chloroquine (0.025 μCi/ml) for 90 min, in the absence or presence of AICAR, compound C and methylamine (MA; added as the neutral hydrochloride compound) at the concentrations as indicated. Values are the mean ± SE of 5 independent experiments. *p<0.05 versus control cells.

Fig. 6. Effect of AICAR, metformin and compound C on autophagic proteolysis in HT-29 cells. Panel A. Left. Effect of AICAR and metformin on AMPK activity. When required, metformin (2 mM) or AICAR (250 μM) was added for 15 min to complete medium before the experiment. Then, the medium was removed and replaced by the adequate medium for 1 h (AICAR, 250 μM; Met, 2 mM). The cells were lysed and the state of phosphorylation of AMPKα was assessed by immunoblotting with the anti-AMPK/phospho-Thr^{172}. Phosphorylation was quantified by the ratio of AMPK-P per total AMPK in arbitrary units. The AMPK-dependent phosphorylation of acetylCoA carboxylase (ACC) was detected by the anti-ACC/phospho-Ser^{79}. Right. Effect of compound C on ACC. Cells were incubated in a glucose-free medium to stimulate the AMPK-dependent ACC phosphorylation. When required, 40 μM compound C (CC) was added. The quantification of ACC phosphorylation was analysed by the ratio of ACC-P per total actin in arbitrary units.

Panel B. Effect of AICAR, metformin (Met) and compound C (CC) on \([^{14}C] \)valine-labelled protein degradation in HT-29 cells in comparison to other known inhibitors of autophagy, amino acids (AA, 4x), 3-methyladenine (3-MA, 10 mM) and interleukin-13 (IL-13, 30 ng/ml). After labelling overnight with radioactive valine, long-lived protein degradation was measured as described in experimental procedures in complete medium (CM) and nutrient-free medium (HBSS). The cell viability was greater than 95% in all conditions. Values are the mean ± SE of three independent experiments. *p<0.05 versus control cells incubated in nutrient-free medium.

Fig. 7. Effect of AMPKCA and AMPKDN on autophagic proteolysis in HT-29 cells. Panel A. Left. Cells were transfected by AMPKCA and AMPKDN constructs and the expression level was detected after SDS-PAGE and immunoblotting with c-myc antibody. (Right) AMPK-dependent phosphorylation of ACC was analyzed in cells transfected with an empty vector, in cells expressing AMPKCA and AMPKDN, respectively. When required AICAR was added at 250 μM. The AMPK-dependent phosphorylation of acetylCoA carboxylase (ACC) was detected by the anti-ACC/phospho-Ser^{79}. The quantification of ACC phosphorylation was expressed by the ratio of ACC-P per total actin in arbitrary units. Panel B. \([^{14}C] \)valine-labelled protein degradation in HT-29 cells expressing AMPKCA and AMPKDN After labelling overnight with radioactive valine, long-lived protein degradation was measured as described in experimental procedures in complete medium (CM) and nutrient-free medium (HBSS). Cells were used 48 h post-transfection. When required, amino acids (AA, 4x); 3-methyladenine (3-MA, 10 mM) and interleukin-13 (IL-13, 30 ng/ml) were added to the chase medium. *p<0.05 versus HT-29 cells expressing AMPKCA incubated in nutrient-free medium.
Fig. 8. Effect of compound C and AMPKDN on autophagic proteolysis in HeLa cells.

Panel A. HeLa cells were co-transfected with the cDNAs encoding GFP-LKB1 and FLAG-STRAD (T) or with control vectors (C). LKB1 and STRAD were detected by immunoblotting with an anti-GFP and an anti-FLAG, respectively, in co-transfected cells. HeLa LKB1/STRAD cells were transfected with the cDNA encoding myc-AMPKDN (T1) or with control vector (C1) The transfected protein was detected by immunoblotting with the anti-c-myc. Panel B. The AMPK-dependent phosphorylation of acetylCoA carboxylase (ACC) was detected by the anti-ACC/phospho-Ser79 in complete medium (CM) and nutrient-free medium (HBSS). The quantification of ACC phosphorylation was expressed by the ratio of ACC-P per total actin in arbitrary units. When required AICAR and CC were added at 250 μM and 40 μM, respectively. Panel C. [14C]valine-labelled protein degradation in HeLa cells, HeLa LKB1/STRAD and HeLa LKB1/STRAD/AMPKDN cells. After labelling overnight with radioactive valine, long-lived protein degradation was measured as described in experimental procedures in complete medium (CM) and nutrient-free medium (HBSS). Cells were used 48 h post-transfection. When required, amino acids (AA, 4x), 3-methyladenine (3-MA, 10 mM) and compound C (CC, 40 μM) were added to the chase medium. Values are the mean ± SE of three independent experiments. *p<0.05 versus control cells and HeLa LKB1/STRAD cells incubated in nutrient-free medium.

Fig. 9. MDC staining of HeLa LKB1/STRAD cells and HeLa LKB1/STRAD/AMPKDN cells. HeLa LKB1/STRAD cells and HeLa LKB1/STRAD/AMPKDN cells were treated for 2 h in appropriate media (3-MA, 10 mM; AA, 4x), and, during the last 10 min, 0.1 µM MDC was added. Only cells expressing GFP-LKB1 were used for MDC counting. The bar represents 5 µm. *p<0.05 versus the control in HBSS alone; **p<0.05 versus HeLa LKB1/STRAD cells incubated in nutrient-free medium.
Fig. 1
AMPK (Thr^{172})

- Control
- AICAR 250 μM
- Metformin 2 mM

Time (min) 20 40 60 80 100

Fig. 2
Fig. 3

AMPK (Thr\textsuperscript{172})

AICAR 250\mu M  Metformin 2 mM

[CC] \mu M

0 10 20 40 0 10 20 40
Fig. 4
Fig. 5

[\textsuperscript{14}C]chloroquine (in/out)
Fig. 6
Fig. 7

A

AMPK<sup>ΔA</sup>-expressing cells

AMPK<sup>DN</sup>-expressing cells

HBSS

Control

AMPK<sup>ΔA</sup>

AICAR

AICAR + AMPK<sup>DN</sup>

Proteolysis (%/h)

CM

HBSS

AA

3-MA

IL-13

ACC (Ser<sup>79</sup>)

Actin

0

0.5

1.0

1.5

2.0

2.5

A.U.

Control

AMPK<sup>ΔA</sup>

Control AICAR

AICAR + AMPK<sup>DN</sup>

Proteolysis (%/h)

CM

HBSS

AA

3-MA

IL-13

Fig. 7
Fig. 8
Fig. 9
AMP-activated protein kinase and the regulation of autophagic proteolysis
Daniel Meley, Chantal Bauvy, Judith H.P.M. Houben-Weerts, Peter F. Dubbelhuis,
Mariette T.J. Helmond, Patrice Codogno and Alfred J. Meijer

J. Biol. Chem. published online September 21, 2006

Access the most updated version of this article at doi: 10.1074/jbc.M605488200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/early/2006/09/21/jbc.M605488200.citation.full.html#ref-list-1