Despite its importance in terms of energy homeostasis, the role of AMP-activated protein kinase in adipose tissue remains controversial. Initial studies have described an anti-lipolytic role for AMP-activated protein kinase whereas more recent studies have suggested the converse. We have thus addressed the role of AMP-activated protein kinase in adipose tissue by modulating AMP-activated protein kinase activity in primary rodent adipocytes using pharmacological activators or by adenoviral expression of dominant negative or constitutively active forms of the kinase. We then studied the effects of AMP-activated protein kinase activity modulation on lipolytic mechanisms. Finally, we analysed the consequences of a genetic deletion of AMP-activated protein kinase α1 Knock-out mice, lipolysis is increased. We present data suggesting that AMP-activated protein kinase activation has a clear anti-lipolytic effect.

AMP-activated protein kinase (AMPK) is a widely expressed serine/threonine kinase which is considered to act as an intracellular sensor of energy. AMPK is a heterotrimeric complex consisting of a catalytic (α) and two regulatory (β and γ) subunits (1). For each subunit, several isoforms have been identified, although the exact function of the different isoforms remains unclear. Numerous studies have shown that AMPK is activated by stresses which increase the AMP/ATP ratio into the cell such as hypoxia, exercise and long term starvation (1,2). AMPK is activated by phosphorylation of the threonine residue 172 within the activation loop of the α catalytic subunit by AMPK Kinase (AMPKK). AMPKK has been recently identified as LKB1, a tumor suppressor mutated in humans with Peutz-Jeger syndrome. This disorder is associated with increased risk of colon, stomach and lung carcinomas (3-5).

The activation of AMPK switches on catabolic pathways that produce ATP and switches off anabolic pathways that consume ATP. Activation of AMPK leads to the phosphorylation of a number of proteins that results in increased glucose uptake and metabolism as well as fatty acid oxidation and simultaneously in an inhibition of hepatic lipogenesis, cholesterol synthesis and glucose production (1). AMPK is also responsible for increased fatty acid oxidation in response to the adipocyte-derived hormones, leptin and adiponectin (6,7). Since AMPK activation would have beneficial metabolic consequences for
diabetic patients, AMPK has emerged as a potential target for the treatment of obesity and type 2 diabetes (8,9). It has been demonstrated that two classes of antidiabetic drugs, metformin and thiazolidinediones could act at least in part through an activation of AMPK in liver and muscle (10,11).

Whereas the function of AMPK in liver and muscle has been well illustrated, its role in adipose tissue remains poorly documented and controversal. Adipose tissue is an important actor for the preservation of energetic homeostasis. The breakdown of triglycerides through lipolysis during fasting is a major function in adipocytes. Previously, Sullivan et al. reported that prior activation of AMPK by 5-aminoimidazole-4-carboxamide-riboside (AICAR) in isolated rat adipocytes inhibits isoproterenol-induced lipolysis (12). However, recently, a study performed in the 3T3-L1 adipocyte cell line has reached the opposite conclusion. Based on the fact that β adrenergic agents activate AMPK activity in adipocytes and that lipolysis is lower in the presence of a dominant negative form of the kinase, it was proposed in this latter study that AMPK stimulates the lipolytic pathway (13).

In order to clarify the role of AMPK, we have modulated the activity of the kinase in primary rodent adipocytes and in 3T3-L1 adipocytes using pharmacological activators or with adenoviruses expressing either dominant negative or constitutively active forms of the kinase and have studied the effects of these manipulations on lipolytic mechanisms. We have also analysed the consequences of genetic deletion of AMPK activity in mouse adipocytes.

EXPERIMENTAL PROCEDURES

Animals - Animal studies were conducted according to the French guidelines for the care and use of experimental animals. Male Sprague-Dawley rats (200-300g body weight) and male CBA mice (25-35 g) were obtained from Charles River laboratory (L'Arbresle, France). All experiments were performed on adipocytes isolated from epididymal fat pads. The AMPK \textsuperscript{a1} Knock-Out (KO) mice were as previously described (14). Epididymal and inguinal fat pads of five-months old wild type (WT) and AMPK \textsuperscript{a1} KO mice were used in this study. Animals were housed in cages at a constant temperature (22 °C) with light from 0700 h to 1900 h and free access to water and laboratory chow (UAR, France). For the fasting/refeeding experiments, rats were fasted for 12 or 24 h and then refed for 1 or 3 h with a high carbohydrate diet (72% carbohydrate, 1% fat and 27 % protein (% energy)).

Adipocyte isolation and determination of fat cell size and number - Mature adipocytes were isolated by collagenase treatment of epididymal fat pads according to Rodbell (15). The diameter of adipocytes was measured using a light microscope fitted with a camera and Perfect-Image software (Clara Vision). A frequency distribution plot of cell diameters was used to determine the mean fat cell diameter and standard deviation about the mean. Both mean fat cell volume and surface area were then determined using standard equations. Mean fat cell weight was calculated from cell volume, assuming that the density of lipid is equal to that of triolein (0.915 g/l). After determination of triglyceride concentration (Kit Infinity triglyceride reagent, Sigma Diagnostics, St Quentin Fallavier, France) in the cell suspension, fat cell number was calculated by dividing the lipid content of the cell suspension by the mean fat cell weight.

3T3-L1 culture - 3T3-L1 cells (a kind gift from Dr. J. Pairault, Paris, France) were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (PAA, Les Mureaux, France) and antibiotics. At confluence, adipocyte differentiation was induced by adding methylisobutylxanthine (100 µM), dexamethasone (0.25 µM), and insulin (1µg/ml) for 2 days. Cells were cultured in high-glucose DMEM supplemented with 10% fetal calf serum and insulin for an additional 5 days and then maintained in high-glucose DMEM only supplemented with 10% calf serum. 3T3-L1 adipocytes were used for experiments 8-10 days after differentiation and treated as described below for mature adipocytes.

Short term incubation of adipocytes and AMPK assay - After isolation, adipocytes (approximately 3 x10 \textsuperscript{6} cells) were incubated in Krebs Ringer Hepes buffer (pH 7.4) supplemented with 2% BSA in a 95/5 % O\textsubscript{2}/CO\textsubscript{2} atmosphere for 30 min. Cells were then washed three times with Hanks buffer to remove BSA and then incubated 1 h in Hanks buffer in a 95/5 % O\textsubscript{2}/CO\textsubscript{2} atmosphere in the presence or absence of the different AMPK activators. Following treatment, adipocytes were disrupted in buffer A, 50mM Hepes pH 7.4, 1mM EDTA,
1mM EGTA, 10% glycerol, 50mM NaF, 5mM sodium pyrophosphate, 1mM DTT and protease inhibitors (Complete protease inhibitor, Roche, Meylan, France) supplemented with 1% Triton X100. The cellular debris were pelleted by centrifugation at 4000g for 15 min at 4°C and the resulting supernatant was recovered, adjusted to 10% PEG 8000 and incubated 45 min at 4°C. Following further centrifugation (18000 g, 15 min), the pellet of proteins was resuspended in buffer A. Aliquots were used to assay the AMPK activity using AMARA peptide in the presence of saturating concentrations of 5’-AMP (200 µM) as described previously (16).

Immunoprecipitation of AMPK complexes - AMPK complexes were immunoprecipitated from 500 µg of adipocyte proteins by incubation with either anti α1, anti α2 or anti α1 + anti α2 antibodies bound to protein G-sepharose beads (Amersham Biosciences, Orsay, France). Recombinant AMPK α1 proteins (AMPK-DN and AMPK-CA) were immunoprecipitated from adipocyte lysates using an anti-Myc (clone 9E10) antibody bound to protein G-sepharose beads (Amersham Biosciences, Orsay, France). Immune complexes were collected by brief centrifugation and washed extensively in buffer A. AMPK activity in the immune complex was determined by phosphorylation of the AMARA synthetic peptide substrate as described (16).

Infection of adipocytes with adenovirus - Adenoviruses encoding a constitutively active form (Ad AMPK-CA) and a dominant negative form (Ad AMPK-DN) of AMPK were as previously described (17). An adenovirus of which the expression cassette contains the major late promoter with no exogenous gene was used as control (Ad Null). Adenoviruses were propagated in HEK 293 cells, purified by cesium chloride density centrifugation and stored at −80°C. Adipocytes (approximately 10⁶ cells) were transduced in 2 ml of DMEM with various titers of adenovirus (from 100 to 200 pfu/cells) for 4 h at 37 °C. Culture medium was subsequently adjusted to 4 ml with DMEM supplemented with 25mM glucose, 1% fetal calf serum, 2% BSA and antibiotics. The efficacy of infection was estimated by the presence of GFP in adipocytes, which is produced from an independent promoter by the Ad AMPK-CA. Infection efficiency was always higher than 50%. Adipocytes were infected for 24 h with the Ad AMPK-DN and for 48 h with the Ad AMPK-CA prior to the lipolysis assay.

Western Blot analysis - Samples were boiled in SDS sample buffer, resolved by SDS-PAGE and transferred to a nitrocellulose membrane (Hybond ECL, Amersham). The membrane was incubated 1 h at room temperature in TBST buffer (10mM Tris-HCl (pH 7.4), 150mM NaCl and 0.05% (vol/vol) Tween 20) containing 5% (wt/vol) low-fat milk powder. Following a 2 h incubation with primary antibody at room temperature, the blots were washed with TBST buffer and incubated with the appropriate second antibody coupled to horseradish peroxidase. After washing, blots were developed using enhanced chemiluminescence (SuperSignal, Pierce). The recombinant AMPK-CA and AMPK-DN proteins contain a N-terminal Myc epitope (Woods et al., 2000) and were detected using an anti-Myc monoclonal antibody (clone 9E10, Roche, Meylan, France). Antiphospho-hormone sensitive lipase (HSL) (Ser565) antibody and total HSL antibody were provided by Pr D.G. Hardie (Dundee, UK) and Dr C. Holm (Sweden) respectively. Antiphospho-Acetyl-CoA carboxylase (ACC) (Ser79) and antiphospho-cAMP response element binding protein (CREB (Ser133)) were obtained from Upstate (Milton Keynes, UK). Antiphospho-AMPK (Thr172) and anti-β actin antibodies were respectively supplied by Cell signaling (New England Biolabs, UK) and Sigma Aldrich (Saint Quentin Fallavier, France). Antibody against perilipin was obtained from Progen (Progen Biotechnik, Heidelberg, Germany). Total ACC was detected using streptavidin conjugated to horseradish peroxidase (Amersham Biosciences, Orsay, France).

Lipolysis assay - Isolated fat cells were infected with AMPK adenovirus or treated with AMPK activators as described above. Adipocytes (10⁶ cells) were then incubated at 37°C for 1 h in 2.5 ml Krebs Ringer bicarbonate buffer pH 7.4 containing 4% BSA with or without 1 µM isoproterenol in an atmosphere of 95/5 % O₂/CO₂ in stoppered Nalgene vials. Prior to lipolysis assay, 3T3-L1 adipocytes were incubated in Krebs Ringer bicarbonate buffer pH 7.4 containing 4% BSA for 3 hrs. Then, they were treated 1 h with AICAR or phenformin followed by 1h treatment with or without 10 µM isoproterenol. Subsequently, 1ml of the incubation medium of rat adipocytes or 3T3-L1 adipocytes was removed, acidified with 100 µl of 30% trichloroacetic acid. The mixture was
vigorously shaken and then centrifuged at 3000 g for 10 min at 4°C. A volume of 700 µl of supernatant was collected and neutralized with 80 µl of 10% KOH and assayed for glycerol content (Glycerol Kit, Enzytec, Diffchamb, France).

**Measurement of cellular ATP content**- Isolated fat cells were infected with AMPK adenovirus or treated with AMPK activators as described above. The incubation medium was then removed and cells were disrupted in perchloric acid solution (PCA 7% v/v final). After centrifugation (3000g, 10 min), the acidic cell extract was neutralized with KOH and the ATP concentration was measured using a spectrophotometric procedure.

**Isolation of lipid droplets** - Lipid droplets were isolated according to Yu et al (18). Briefly, adipocytes were resuspended in 2 ml of disruption buffer (25mM Tris HCl pH 7.4, 100mM KCl, 1mM EDTA, 5mM EGTA and protease inhibitors). Cells were disrupted by nitrogen cavitation at 800 psi for 10 minutes at 4°C. The cavitate was collected, mixed with an equal volume of disruption buffer containing 1.08 M sucrose and overlaid sequentially with 2 ml of 0.27 M sucrose buffer, 2 ml of 0.135 M sucrose and 2 ml of "top" buffer (25mM Tris HCl pH 7.4, 1mM EDTA, and 1mM EGTA). Following centrifugation at 150000 g for 90 min, different fractions (1.5 ml each) were collected from the top (fraction containing lipid droplets) to the bottom of the tube (cytosol, microsome and nuclei). The lipid droplets were then washed with top buffer and centrifuged at 3500 g for 15 min at 10°C.

**Protein assay** - Protein concentration was measured according to Bradford with BSA as standard.

**Statistical analysis** - Results are expressed as mean ± SE. The level of significance in the difference between groups was calculated by Student’s unpaired t-test except for AMPK activity (Fig.1 and 8) and lipolysis (Fig.8) in which statistical significance was calculated by paired t-test.

**RESULTS**

**Adipocytes express AMPK complexes containing the α1 catalytic subunit.** Since AMPK complexes contain one of the two catalytic subunits (α1, or α2), which respond differently to various stimuli (6,19), we first determined the nature of the catalytic subunit accounting for AMPK activity in adipose tissue. Isolated rat adipocytes were incubated for 1 h in the absence or presence of AICAR, a cell permeable activator of AMPK. Western-blot analysis of adipocyte lysates revealed the preponderance of the α1 catalytic subunit (Fig 1A). In order to confirm this trend, AMPK complexes were immunoprecipitated from lysates, using antibodies against α1 or α2 subunits, or a mixture of α1 and α2 to measure total activity. Total AMPK activity was stimulated 2-fold by AICAR (Fig. 1B). In basal as well as in AICAR-stimulated conditions, the activity of α1 containing AMPK complexes is predominant and accounts for more than 90% of total AMPK activity measured (Fig. 1B and C). Although the activity of α2 containing AMPK complexes is extremely low, it is also significantly stimulated by AICAR.

**cAMP-generating agents and fasting induce AMPK activity in adipocytes.** Treatment of isolated adipocytes with isoproterenol, a β-adrenergic agonist or BRL 37344, a specific agonist of the β3 adrenergic receptor is followed by a robust increase of total AMPK activity (Fig. 2A) as previously described (13,20). Forskolin, which activates adenylate cyclase independently of the β-agonist receptor and Bt2cAMP have also a stimulatory effect on AMPK activity. Thus AMPK activity is stimulated under conditions of increased cAMP concentrations. Since fasting induces a β-adrenergic stimulation of adipocytes, we have compared AMPK activity in the epididymal fat pad of fasted and refed rats. AMPK activity is 2-fold higher in fasted compared to refed rats (Fig. 2B). This is associated with an increase of the phosphorylation state of Ser79 of ACC, a known target of AMPK whereas total ACC content is not modified in these conditions.

**Activation of AMPK activity inhibits lipolysis in adipocytes.** Since AMPK activity is increased during fasting, a state in which lipolysis is strongly induced, we next investigated the relationship between AMPK and lipolytic activity. In isolated rat adipocytes the lipolytic rate is increased ten-fold in the presence of isoproterenol (Fig. 3A). Short-term incubations of adipocytes with AICAR or phenformin, a closely-related analog of metformin, activate AMPK as shown by the increase of the phosphorylation state of AMPK Thr172 (Fig. 3B) and strongly inhibit the isoproterenol-induced lipolysis (Fig. 3A) in a parallel manner. In order to demonstrate that the inhibitory effects
of AICAR and phenformin on lipolysis are not linked to an impairment of the protein kinase A (PKA) pathway, we examined whether the presence of AICAR or phenformin depleted the ATP concentration in adipocytes. As shown previously by others (21,22), AICAR does not modify ATP concentration in adipocytes (48 ± 3 nmol of ATP/10⁶ cells in non treated adipocytes vs 45.3 ± 2.7 in AICAR-treated adipocytes). Phenformin decreases ATP concentration in adipocytes (34.3 ± 0.6 nmol of ATP/10⁶ cells). However, neither AICAR nor phenformin interfere with the phosphorylation on serine 133 of the cAMP responsive element binding protein (CREB) in the presence of isoproterenol. This demonstrates that activation of lipolysis is not impaired in the presence of these compounds (Fig. 3C).

AICAR and phenformin are not specific activators of AMPK. Thus in order to verify that their inhibitory effects on the lipolytic pathway were indeed mediated by AMPK, we overexpressed a constitutively active form of AMPK (AMPK-CA) in adipocytes. The AMPK-CA corresponds to a truncated catalytic α₁ subunit in which the phosphorylation site (Thr172) has been mutated to an acidic amino acid in order to mimic phosphorylation (17). Overexpression of AMPK-CA does not affect adipocyte viability since after 36h of infection the number of adipocytes is very similar in Ad Null (0.71±0.11x10⁶) or in Ad AMPK-CA (0.84±0.02x10⁶) infected adipocytes. ATP concentration into adipocytes is not statistically modified in the presence of the AMPK-CA (82.0±0.65 nmol of ATP/10⁶ cells in Ad null treated cells vs 71.6±4.1 in AMPK-CA treated cells). Overexpression of the AMPK-CA in mature adipocytes, leads to an increased AMPK activity similar to that measured in adipocytes treated for 1h with AICAR and induces the phosphorylation of ACC on Ser79 to the same extent than AICAR (Fig. 4A). This specific increase of AMPK activity inhibits the isoproterenol-induced lipolysis (Fig. 4B). This confirms the results obtained using AICAR and phenformin and shows that the antilipolytic effect of these agents was mediated at least in part by AMPK.

**Inhibition of AMPK activity using a dominant negative form of AMPK increases lipolysis.** The results shown above suggest that when AMPK is activated it inhibits the lipolytic pathway. If this is true, blockade of AMPK activity in adipocytes should increase the isoproterenol-induced lipolytic rate. In order to test this hypothesis, we overexpressed a dominant negative form of the α₁ subunit of AMPK (AMPK-DN) in adipocytes (17). The overexpression of AMPK-DN does not affect cell viability after 24 h of infection (1.10±0.01 10⁶ adipocytes in Ad null conditions vs 1.16±0.05 10⁶ adipocytes in Ad AMPK-DN conditions) and ATP concentration (62.3±9.6 nmol of ATP/10⁶ Ad null cells vs 55.10±2.2 nmol of ATP/10⁶ Ad AMPK-DN cells). The dominant negative form of the α₁ subunit is effective since it impairs the activation of AMPK by AICAR and isoproterenol as well as the phosphorylation of ACC on Ser79 in response to AICAR and isoproterenol (Fig. 5A). Finally, the inhibition of AMPK activity, which has a small and not significant inhibitory effect on basal lipolysis, stimulates the isoproterenol-induced lipolysis (Fig. 5B) confirming that in lipolytic conditions, the role of AMPK is to limit the lipolytic rate.

**Phosphorylation of HSL by AMPK reduces its translocation towards the lipid droplet.** HSL is the key enzyme controlling lipolysis in adipocytes. Activity of HSL is regulated acutely by several mechanisms, including reversible phosphorylation by a number of protein kinases and translocation from the cytosol to the surface of the lipid droplet (23). It has been previously reported that phosphorylation of HSL on Ser565 has no effect on HSL activity per se but abolishes the activating phosphorylation of protein kinase A on the adjacent Ser563 (24) thus decreasing in fine HSL activity.

We have thus analysed the effects of AMPK stimulation on HSL phosphorylation and translocation to the lipid droplet. Treatment of adipocytes with AICAR or expression of the AMPK-CA induces the phosphorylation of HSL on Ser565 (Fig. 6A). A ten-minute incubation of adipocytes in the presence of isoproterenol induces the translocation of HSL from the cytosol (Fig. 6B lane 1 vs 2) to the lipid droplet (Fig. 6B lane 5 vs 6). Prior activation of AMPK by treatment with AICAR or phenformin strongly impaired the mobilization of HSL to the droplet (Fig. 6B lane 2 vs 3 and 4; lane 6 vs 7 and 8). In order to assess that the default in HSL translocation after AICAR treatment is indeed mediated by AMPK, we overexpressed the AMPK-DN form to block endogenous AMPK activity. In Ad null infected cells, isoproterenol induces the translocation of HSL to the lipid droplet and AICAR inhibits this process.
Inhibition of AMPK activity totally reverses the effect of AICAR on HSL translocation (Fig. 6C).

**AMPK activation in 3T3-L1 adipocytes inhibits lipolysis and translocation of HSL to the lipid droplet.** Since opposite results of those presented here have been obtained in 3T3-L1 adipocytes (13), we have tried to repeat some of our experiments in this cell line. Treatment of 3T3-L1 for 1h with 500 µM AICAR or 100 µM phenformin induces a two-fold increase in AMPK activity (0.33 ±0.001 pmol of ATP incorporated /µg of proteins/min in control cells, 0.58±0.04 pmol of ATP incorporated /µg of proteins/min in AICAR treated cells, 0.60±0.03 pmol of ATP incorporated /µg of proteins/min in phenformin treated cells). As previously observed in mature rat adipocytes (Fig. 3A), prior activation of AMPK by 500 µM AICAR or 100 µM phenformin decreases the isoproterenol-induced lipolysis (Fig. 7A). Finally, treatment of 3T3-L1 with AICAR impaired the translocation of HSL induced by isoproterenol to the lipid droplet (Fig. 7B). Altogether, these results suggest that as previously demonstrated in mature adipocytes, the activation of AMPK inhibits the lipolytic pathway in 3T3-L1 adipocytes.

**Adipocyte metabolism in AMPK α1 knock out mice.** Since the activity of the α containing AMPK complex is predominant in adipose tissue (Fig. 1), the importance of AMPK in adipose tissue metabolism should be revealed in AMPK α1 knock-out (KO) mice. Since in a number of KO animal studies, compensation by a close-relative isoform is often observed, we have first measured α1 and α2 AMPK activity in adipocytes of wild type (WT) and AMPK α1 KO mice. As shown previously in rats (Fig.1), AMPK α1 activity is the predominant activity measured in adipocytes of WT mice (Fig.8A). In adipose tissue of AMPK α1 KO mice, AMPK α1 activity is severely blunted and no compensatory increase in AMPK α2 is observed (Fig.8A). The body weight of the AMPK α1 KO mice is not statistically different from that of wild type (WT) mice although it has a tendency to be lower (Table 1). However, the weight of different adipose tissue depots is strongly reduced in the KO animals (Table 1). We then measured the size of adipocytes in both groups and found that the mean diameter is decreased in the KO mice (57.96 µm vs 35.6 µm, control vs KO) (Fig.8B). The lipolytic rate is increased two-fold in the presence of isoproterenol in adipocytes of WT mice, an effect which is inhibited when the cells are pretreated with AICAR (Fig.8C). In AMPK α1 KO mice, basal lipolysis as well as isoproterenol-induced lipolysis is increased 2 fold in adipocytes when compared to WT animals and AICAR has no inhibitory effect. This in vivo model confirms the potential anti-lipolytic role of AMPK during lipolysis.

**DISCUSSION**

AMPK became a burning issue when it was discovered that its activation could have beneficial effects in the metabolic syndrome, through an insulin-independent increase in muscle glucose utilization, decreased hepatic glucose production and increased fatty acid oxidation in both muscle and liver. Adipose tissue is a major component of energy homeostasis and a key player in the regulation of insulin sensitivity through fatty acid release (lipolysis) and hormone secretion. Understanding the role of AMPK in adipocytes is thus crucial for a comprehensive view of beneficial/detrimental effects of AMPK activation.

Considering the importance of this issue and the controversial results found in the literature, we decided to investigate the exact function of AMPK on lipolysis using various in vivo and in vitro approaches. We firstly established that AMPK α1 is the predominant subunit expressed in adipocytes. Whether this has a functional significance is presently unclear although it can be emphasized that AMPK complexes containing this isoform are much less sensitive to AMP (25). Secondly, we demonstrated as others (13,20) that cAMP and cAMP-generating agents activate AMPK in adipocytes although the exact mechanism remains unknown. We have confirmed a previous study showing that AICAR strongly inhibits the lipolytic rate induced by β-agonists in primary adipocytes. Furthermore, we have shown that another AMPK-activating agent, phenformin which has a chemical structure totally unrelated to AICAR has a similar effect. The use of adenovirus-mediated expression of dominant positive and negative forms of the kinase in adipocytes has contributed to demonstrate the inhibitory role of AMPK activation on lipolysis. These results are at variance with those of Yin et al who have reached opposite conclusions in 3T3-L1 cells.
Their main argument is based on the fact that adenoviral expression of a dominant negative form of the \(\alpha_2\) subunit of the kinase inhibits isoproterenol-induced lipolysis. However, it must be pointed out that in their crucial experiments using this mutant form, AMPK activity was not measured and it is thus difficult to conclude concerning the reality of the modulation of AMPK activity in these experiments. Using the same cell-line, we demonstrated that treatment with AICAR and phenformin induces AMPK activity and strongly impairs the isoproterenol-induced lipolysis (Fig. 7).

We have addressed the functional significance of AMPK modulation in adipocytes. In vivo, AMPK activity is indeed increased by fasting (Fig. 2), a situation concomitant with lipolysis activation. More importantly in mice lacking the predominant \(\alpha_1\) subunit isoform (13), we have observed (i) that the size of adipocytes is considerably reduced (ii) that basal and isoproterenol-stimulated lipolysis in these small cells is higher than that of larger control adipocytes and (iii) that AICAR has no inhibitory effect on lipolysis, all results compatible with an inhibitory effect of AMPK on the lipolytic rate. This is in agreement with in vivo studies showing an anti-lipolytic effect of AICAR (26).

By which mechanism could AMPK inhibit the lipolytic rate? Since AMPK activation is associated in adipocytes with an increase in glucose uptake (27), it could augment \(\alpha\)-glycerophosphate generation and fatty acid re-esterification. However, the extent of glucose transport stimulation is rather modest. Initial studies have shown that AICAR induces the phosphorylation of HSL on Ser565, precluding the further activating phosphorylation on Ser563 by protein kinase A (24). However, it was then demonstrated that Ser659 and Ser660 rather than Ser563 were probably the true targets of protein kinase A-mediated HSL activation (28). Increased lipolysis is due to HSL activation but also to its translocation to the lipid droplet. A mutation of Ser565 to alanine abolishes the translocation of HSL to the lipid droplet in 3T3-L1 cells casting further doubts on an inhibitory function of an AMPK-mediated phosphorylation on Ser565 (29). Using subcellular fractionation, we have shown here in rat adipocytes and in 3T3-L1 cells that translocation of endogenous HSL to the lipid droplet, a major requirement for an active lipolysis is strongly reduced by AMPK activation, a phenomenon concomitant with increased phosphorylation of Ser565 on HSL. Inhibition of AMPK activity by the AMPK-DN form totally prevents the mislocalization of HSL observed with AMPK activators emphasizing the involvement of AMPK in this process. The discrepancy with the results of Su et al. showing that the absence of phosphorylation at Ser565 precludes HSL translocation is not clear but could be due to the fact that a serine to alanine mutation alters HSL conformation in such a way that it affects the translocation process (29). Alternatively, AMPK could phosphorylate and modulate the function of another protein involved in HSL translocation such as perilipin. This obviously requires further clarification.

The current study thus demonstrates that activation of AMPK reduces fatty acid release in rodent adipocytes. It is difficult to speculate on the cellular advantage of such an effect but it could represent as suggested by Moule & Denton (20) a feedback mechanism allowing to limit lipolysis and the intracellular concentrations of fatty acids which are potentially toxic to the cell. In any case, and if these studies can be extended to humans, activation of AMPK in adipose tissue, by limiting the concentration of circulating fatty acids could be extremely beneficial in pathologies such as obesity and type 2 diabetes.
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ABBREVIATIONS: ACC : Acetyl-CoA carboxylase, AICAR : 5-aminoimidazole-4-carboxamide-riboside, AMPK : AMP-activated protein kinase, CREB : cAMP responsive element binding protein, HSL : hormone sensitive lipase, KO : knock-out, WT : wild type, PKA : protein kinase A.
REFERENCES

1. Hardie, D. G. (2003) *Endocrinology* **144**, 5179-5183
2. Carling, D. (2004) *Trends Biochem Sci* **29**, 18-24
3. Woods, A., Johnstone, S. R., Dickerson, K., Leiper, F. C., Fryer, L. G., Neumann, D., Schlattner, U., Wallimann, T., Carlson, M., and Carling, D. (2003) *Curr Biol* **13**, 2004-2008
4. Shaw, R. J., Kosmatka, M., Bardeesy, N., Hurley, R. L., Witters, L. A., DePinho, R. A., and Cantley, L. C. (2004) *Proc Natl Acad Sci U S A* **101**, 3329-3335
5. Hawley, S. A., Boudeau, J., Reid, J. L., Mustard, K. J., Udd, L., Makela, T. P., Alessi, D. R., and Hardie, D. G. (2003) *J Biol* **2**, 28
6. Minokoshi, Y., Kim, Y. B., Peroni, O. D., Fryer, L. G., Muller, C., Carling, D., and Kahn, B. B. (2002) *Nature* **415**, 339-343
7. Yamauchi, T., Kamon, J., Minokoshi, Y., Ito, Y., Waki, H., Uchida, S., Yamashita, S., Noda, M., Kita, S., Ueki, K., Eto, K., Akanuma, Y., Frooguel, P., Foufelle, F., Ferre, P., Carling, D., Kimura, S., Nagai, R., Kahn, B. B., and Kadowaki, T. (2002) *Nat Med* **8**, 1288-1295
8. Winder, W. W., and Hardie, D. G. (1999) *Am J Physiol* **277**, E1-10
9. Ruderman, N., and Prentki, M. (2004) *Nat Rev Drug Discov* **3**, 340-351
10. Zhou, G., Myers, R., Li, Y., Chen, Y., Shen, X., Fenyk-Melody, J., Wu, M., Ventre, J., Doebber, T., Fujii, N., Musi, N., Hirshman, M. F., Goodyear, L. J., and Moller, D. E. (2001) *J Clin Invest* **108**, 1167-1174
11. Fryer, L. G., Parbu-Patel, A., and Carling, D. (2002) *J Biol Chem* **277**, 25226-25232
12. Sullivan, J. E., Brocklehurst, K. J., Marley, A. E., Carey, F., Carling, D., and Beri, R. K. (1994) *FEBS Lett* **353**, 33-36
13. Jorgensen, S. B., Viollet, B., Andreelli, F., Frosig, C., Birk, J. B., Schjerling, P., Vaulont, S., Richter, E. A., and Wojtaszewski, J. F. (2004) *J Biol Chem* **279**, 1070-1079
14. Rodbell, M. (1964) *J Biol Chem* **239**, 375-380
15. Corton, J. M., Gillespie, J. G., Hawley, S. A., and Hardie, D. G. (1995) *Eur J Biochem* **229**, 558-565
16. Merrill, G. F., Kurth, E. J., Hardie, D. G., and Winder, W. W. (1997) *Am J Physiol* **273**, E1107-1112
17. Yeaman, S. J. (2004) *Biochem J* **379**, 11-22
18. Garton, A. J., Campbell, D. G., Carling, D., Hardie, D. G., Colbran, R. J., and Yeaman, S. J. (1989) *Eur J Biochem* **180**, 229-245
19. Salt, I., Celler, J. W., Hawley, S. A., Prescott, A., Woods, A., Carling, D., and Hardie, D. G. (1998) *Biochem J* **334** (Pt 1), 177-187
20. Bergeron, R., Previs, S. F., Cline, G. W., Perret, P., Russell, R. R., 3rd, Young, L. H., and Shulman, G. I. (2001) *Diabetes* **50**, 1076-1082
FIGURE LEGENDS

Fig. 1. Characterization of AMPK catalytic subunits in rat adipocytes. Isolated rat adipocytes were treated for 1 h in the absence or presence of 500 µM AICAR. (A) Western blot analysis of \( \alpha_1 \) and \( \alpha_2 \) catalytic subunits and \( \beta \) actin in adipocyte lysates. Identical amounts of the each lysate were loaded twice. The membrane was then cut and hybridized separately with \( \alpha_1 \) or \( \alpha_2 \) antibody. Hybridization with the second antibody and revelation by chemoluminescence were the same for the two membranes. (B) Total AMPK activity measured after immunoprecipitation with a combination of \( \alpha_1 \) and \( \alpha_2 \) antibodies. (C) AMPK \( \alpha_1 \) and AMPK \( \alpha_2 \) activities measured after separate immunoprecipitation with specific \( \alpha_1 \) or \( \alpha_2 \) antibodies. AMPK was immunoprecipitated and activity present in the immune complexes was measured by phosphorylation of the AMARA peptide. Activities shown are the mean ± SE from three independent experiments. ** shows statistical difference at p<0.01 when compared to conditions in absence of AICAR.

Fig. 2. Effects of activators of the \( \beta \) adrenergic pathway and starvation on AMPK activity in rat adipocytes. (A) Isolated rat adipocytes were treated for 1 h in the presence of 1µM isoproterenol, 10 µM of BRL 37344 (a specific agonist of the \( \beta_3 \) adrenergic receptor), 100 µM of forskolin or 100 µM of Bt_2cAMP. AMPK activity was measured as described in the Experimental Procedures. Activities shown are the mean ± SE from 5 independent experiments. * and ** show statistical differences at p<0.05 and p<0.01 respectively when compared to basal conditions. (B). Rats were fasted for 12 h or 24 h. A subgroup of rats fasted for 24 h was then refed with a high carbohydrate (HC) diet for 1 h or 3 h. Epididymal adipose tissue was sampled and immediately frozen in liquid nitrogen for assay of AMPK activity. Lysates obtained from adipose tissue were analysed for phosphorylation of Ser79 of ACC and for total ACC content. Activities shown are the mean ± SE from two independent experiments with 5 animals in each group. *** shows statistical difference at p<0.001 when compared to fasted conditions.

Fig. 3. Effects of AICAR and phenformin on AMPK activation and lipolysis in isolated rat adipocytes. (A) Adipocytes were pre-incubated 1 h in the absence or presence of 500 µM AICAR or 100 µM phenformin. Lipolysis was then stimulated by the addition of 1 µM isoproterenol during 1 h. Results are the mean ± SE from 5 independent experiments. ** shows statistical difference at p<0.01 when compared to conditions with isoproterenol. (B) Isolated rat adipocytes were treated for 1 h in the absence or presence of 500 µM AICAR or 100 µM phenformin. Adipocyte lysates were then analysed for phosphorylation of Thr172 of AMPK and for total \( \alpha_1 \) content. (C) Adipocytes were treated as described in (A) and lysates were analysed by Western blot for phosphorylation of Ser133 of CREB and for \( \beta \) actin.

Fig. 4. Effect of a constitutively active form of AMPK on lipolysis in rat adipocytes. Isolated rat adipocytes were infected with 100 pfu/cell of Ad Null or Ad AMPK-CA. Ad Null-infected adipocytes were treated or not with 500 µM AICAR for 1h. (A) Cell lysates of adipocytes harvested 48 h post-infection were analysed for the phosphorylation of Ser79 of ACC and for total ACC content AMPK activity was measured as described before. Activities shown are the mean ± SE from three independent experiments. * shows statistical difference at p<0.05 when compared to Ad Null. (B) 48 h after infection, adipocytes were incubated for 1 h in the absence or presence of 1 µM isoproterenol. Lipolysis was measured as described in the experimental procedures. Results are the mean ± SE from 3 independent experiments. * shows statistical difference at p<0.05 when compared to adipocytes infected with the Ad Null.

Fig. 5. Effect of a dominant negative form of AMPK on lipolysis in rat adipocytes. Isolated rat adipocytes were infected for 24 h in the presence of 200 pfu/cell of Ad Null or Ad AMPK-DN. Cells were then incubated for 1 h in the absence or presence of 500 µM AICAR or 1 µM isoproterenol. (A) After 24 h, cell lysates were analysed for the phosphorylation of ACC on Ser79 and for total ACC content. AMPK activity was measured as described before. Activities shown are the mean ± SE from three independent experiments. * shows statistical difference at p<0.05 when compared to Ad-Null.
infected adipocytes treated with AICAR or isoproterenol. (B) After 24 h of infection with the AMPK-DN adenovirus, adipocytes were treated with 1 µM isoproterenol for 1 h to activate lipolysis. Results are the mean ± SE from 2 independent experiments. * shows statistical difference at p<0.05 when compared to adipocytes infected with the Ad Null.

Fig. 6. Effect of AMPK on HSL phosphorylation state and translocation. (A) Isolated rat adipocytes were incubated for 1 h in the absence or presence of 500 µM AICAR or infected for 48 h in the presence of 100 pfu/cell of Ad AMPK-CA or Ad Null. Cell lysates of adipocytes were then analysed for the phosphorylation of HSL on Ser565 (pHSL) and for total HSL (tHSL). These blots are representative of 3 separate experiments. (B) Adipocytes were pre-incubated 1 h in the absence or presence of 500 µM AICAR or 100 µM phenformin then 1 µM isoproterenol was added for 10 min. Lipid droplets and cytosol fractions were isolated as described in the experimental procedure section. 10 µg of proteins from the lipid droplet fraction and 10 µg of cytosolic proteins were separated by electrophoresis and analysed for HSL and perilipin expression using specific antibodies. These blots are representative of three independent experiments. (C) Isolated adipocytes were infected for 24h with 200 pfu/cell of Ad Null or Ad AMPK-DN. Cells were treated 1h in the absence or presence of AICAR and then isoproterenol was added for 10 min. Lipid droplets were isolated and analysed for their content in total HSL (tHSL) and perilipin. This blot is representative of two independent experiments.

Fig. 7. Effect of AICAR treatment on lipolysis and HSL translocation in 3T3-L1 adipocytes. 3T3-L1 adipocytes were used for experiments 8 to 10 days after differentiation. (A) 3T3-L1 adipocytes were incubated in Krebs Ringer bicarbonate buffer pH 7.4 containing 4% BSA for 3 h. Prior the lipolysis assay, cells were treated or not with 500 µM AICAR or 100 µM phenformin. Then they were treated for 1 h with or without 10 µM isoproterenol. Results are the mean ± SE from 2 independent experiments. ** shows statistical difference at p<0.01 when compared to conditions with isoproterenol. (B) 3T3-L1 adipocytes were pre-incubated 1 h in the absence or presence of 500 µM AICAR then 1µM isoproterenol was added for 10 min. Lipid droplets were isolated as described in the experimental procedure section. 10 µg of proteins from the lipid droplet fraction were analysed for total HSL (tHSL) and perilipin expression using specific antibodies. This blots is representative of two independent experiments.

Fig. 8. Analysis of adipocyte cell size and lipolysis in the adipose tissue of AMPK α1 KO mice. (A) ω1 and ω2 AMPK activities were measured from lysates of inguinal fat pads of WT and AMPK α1 KO mice after immunoprecipitation. *** shows statistical difference at p<0.001 when compared to AMPK α1 activity measured in adipocytes of WT mice. (B) Adipocytes were isolated from the inguinal and epididymal fat pads of four WT and four AMPK α1 KO mice and pooled for each fat pad. The number and the size of adipocytes were determined as described in the experimental procedure section. The results presented show the frequency distribution of adipocyte cell size in WT and KO mice (C). Adipocytes from epididymal fat pads were pre-incubated 1 h in the absence or the presence of 500 µM AICAR then 1 µM isoproterenol was added during 1 h to activate lipolysis. Results are the mean ± SE from 2 independent experiments. In each independent experiment, the adipose tissues of 4 mice were pooled. Each experimental condition has been performed in triplicate. ** shows statistical difference at p<0.01 when compared to conditions with isoproterenol. ## shows statistical difference at p<0.01 when compared to to similar lipolytic conditions in WT mice.
Table 1: Metabolic characteristics of the AMPK β1 KO mice. Wild type and KO mice used are 5 months old. Values are the mean ± SE. *, ** statistical differences at p<0.05 and p<0.01 respectively when compared to WT animals.

|                      | WT (n=8)     | β1 AMPK KO (n=8) |
|----------------------|--------------|------------------|
| Body weight (g)      | 33.58 ± 1.41 | 30.1 ± 0.98      |
| Inguinal fat pads (mg)| 383.8 ± 39.1 | 163.5 ± 4.8 *    |
| Epididymal fat pads (mg) | 595.9 ± 60.2 | 272.7 ± 29.8 **  |
Figure 1

**Figure 1**

A. Western blot analysis of β-actin expression in the presence of AICAR.

B. Bar graph showing increased AMPK activity in the presence of AICAR.

C. Bar graph showing AMPK activity in the presence of AICAR and AICAR + AICAR.
Figure 2

Panel A: Graph showing AMPK activity (pmol ATP incorporated/µg prot/min) with different treatments: - Isoproterenol, BRL 37344, FSK, and Bt2AMPc. The x-axis represents different treatments, and the y-axis represents AMPK activity. There are significant differences indicated by asterisks (*, **).

Panel B: Western blot analysis showing pACC (Ser79) and total ACC levels in fasted and refed states at 12 h, 24 h, 1 h, and 3 h. The graphs show AMPK activity (pmol ATP incorporated/µg prot/min) with significant differences indicated by asterisks (***, ****).
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7

A

Lipolysis (µg/glycerol/mg prot)

- - Isoproterenol

- - AICAR Phenformin

B

Lipid droplet

- - AICAR Isoproterenol

t HSL

Perilipin
Figure 8
