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AMPK activation regulates apoptosis, adipogenesis, and lipolysis by eIF2α in adipocytes

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Abstract

AMP-activated protein kinase (AMPK) is a metabolic master switch regulating glucose and lipid metabolism. Recently, AMPK has been implicated in the control of adipose tissue content. Yet, the nature of this action is controversial. We examined the effect on F442a adipocytes of the AMPK activator-AICAR. Activation of AMPK induced dose-dependent apoptotic cell death, inhibition of lipolysis, and downregulatation key adipogenic genes, such as peroxisome proliferator-activated receptor (PPARγ) and CCAAT/enhancer-binding protein alpha (C/EBPα). We have identified the α-subunit of the eukaryotic initiation factor-2 (eIF2α) as a target gene which is phosphorylated following AICAR treatment. Such phosphorylation is one of the best-characterized mechanisms for downregulating protein synthesis. 2-Aminopurine (2-AP), an inhibitor of eIF2α kinases, could overcome the apoptotic effect of AICAR, abolishing the reduction of PPARγ and C/EBPα and the lipolytic properties of AMPK. Thus, AMPK may diminish adiposity via reduction of fat cell number through eIF2α-dependent translation shutdown.

Keywords: AMPK; AICAR; Adipocyte; F442a; eIF2α; PPARγ; C/EBPα; 2-Aminopurine; Apoptosis; Lipolysis

AMPK is a serine/threonine protein kinase, which is activated by cellular stresses that deplete ATP [1]. Under conditions of nutritional and metabolic stress AMPK responds to increases in the AMP/ATP ratio by switching off ATP-consuming pathways and switching on pathways for ATP generation [2]. It is a heterotrimeric protein comprised of α catalytic subunit and two regulatory subunits, β and γ [3,4]. The subunit contains the kinase domain and phosphorylation at threonine 172 of this subunit results in increased AMPK activity [5].

In recent years, the study of AMPK in mammalian organisms has led to an understanding of its role in regulating cellular energy balance. Identification of physiological processes in which the enzyme plays a crucial role indicates the control of body fat stores by AMPK [6,7].

Studies by Winder et al. provided the first evidence that AMPK activation may diminish adiposity, demonstrating a sharp reduction in epididymal and retroperitoneal fat pads in rats after treatment with the AMPK activator AICAR [8,9].

Subsequent studies show the ability of the adipocyte-derived hormones leptin and adiponectin, adrenergic agonists, and metformin to activate AMPK in adipocytes. Furthermore, reduction of fat mass by these mediators was achieved, at least in part, via peripheral AMPK activation [6].

In principle, there are two pathways to diminish adipose tissue mass: one is by a reduction of adipocyte volume, and the other, by reduction of adipocyte number [10,11]. The decrease of the adipose tissue mass is usually attributed to loss of stored lipids by lipolysis rather than through fat cell loss. Yet, attempts to attribute to AMPK a role in the lipolytic process have yielded contradictory results. While Yin et al. [12] demonstrated the essential role of AMPK in lipolysis, others have claimed the opposite—that

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AMPK activity is anti-lipolytic [13]. Decrease of adipose tissue in human could result from loss of fat cells through programmed cell death [14,15]. Following hormonal and metabolic stimulators of peripheral AMPK such as leptin and severe nutrient deprivation can mediate fat mass depletion through apoptotic pathways [16,17].

In response to environmental insult, a family of protein kinases phosphorylates eIF2α to alleviate cellular injury or alternatively induces apoptosis. Phosphorylation of eIF2α reduces global translation, allowing cells to conserve resources and to initiate a reconfiguration of gene expression to effectively manage stress conditions [18]. Accompanying this general protein synthesis control, eIF2α phosphorylation induces translation of specific mRNAs, that assist in the regulation of genes involved in metabolism, the redox status of the cells, and apoptosis [19–22].

To elucidate the mechanism by which AMPK may suppress adiposity, we studied the effect of AICAR on F442a adipocyte cells. We report that AICAR triggers apoptotic cell death and phosphorylates eIF2α in a dose-dependent manner. Suppression of AICAR phosphorylation of eIF2α by 2-AP, a specific kinase inhibitor [1], could overcome the apoptotic effects. Furthermore, 2-AP could moderate reduction of adipogenic markers C/EBPα and PPARγ, and suppress the anti-lipolytic effect. These results suggest that AMPK may modulate adipose tissue at least in part, via inhibition of eIF2α translation.

Materials and methods

Cells. 3T3-F442a fibroblasts (kindly provided by Prof. Menachem Rubinstein) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) bovine serum (BS), 1% (v/v) penicillin–streptomycin (10,000 U/ml penicillin, 10,000 μg/ml streptomycin in 0.85% saline) at 37 °C in 95% air, 5% CO_2. Two days after reaching confluence, the cells were induced to differentiate into adipocytes by switching to 10% fetal calf serum (FCS) supplemented with 5 μg/ml insulin (Sigma). After 2 days, the medium was replaced with DMEM/10% FCS, which was changed every 2 days thereafter until analysis.

Cell differentiation was monitored by examining the cultures with phase-contrast microscope. AICAR was obtained from Toronto Research Chemicals (TRC). Antibodies for immunoblotting of P-eIF2α, PARP, AMPK, and P-AMPK were gained from Cell Signaling, C/EBPα, PPARγ, and actin were gained from Santa Cruz Biotechnology (Santa Cruz, CA).

Immunoblot analysis. Cells were washed twice with a phosphate-buffered saline (PBS) and then lysed in RIPA buffer (0.1% SDS, 1% Nonidet P-40 (NP40), 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 2 mg/ml leupeptin, and 2 mg/ml aprotinin). Cell lysates were cleared by centrifugation (20,000*g*, 10 min, 4 °C). Aliquots of the clarified lysates containing 30 mg protein were denatured in Laemmli sample buffer (6% SDS 30% glycerol, 0.02% bromophenol blue, 200 mM Tris–HCl (pH 6.8), and 250 mM β-mercaptoethanol) at 95 °C for 5 min. The samples were resolved by SDS–PAGE (10% acrylamide) and blotted onto nitrocellulose membrane. Non-specific binding in a Western blot analysis was prevented by immersing the membranes in blocking buffer (5% non-fat dried milk in Tris-buffered saline–Tween 20 (TBS–T)) for 2 h at room temperature. The membranes were then exposed to the indicated antibodies diluted 1:1000 for 1 h at room temperature. The blots rinsed in TBS–T and then incubated with horseradish peroxidase-conjugated goat anti-mouse antibodies 1:10,000 for 1 h at room temperature. Antibody–antigen complexes were visualized by detecting enhanced chemiluminescence with X-ray film, according to the manufacturer's instructions.

Annexin V staining. Apoptosis was evaluated in cells treated with AICAR or vehicle for 48 h, using the Annexin V FITC Detection Kit according to the manufacturer's instruction (Oncogene Research Products, Cambridge, MA). Briefly, both attached and floating cells were collected, washed with cold PBS, re-suspended at a density of 5×10^5 ml in 0.5 ml RPMI-10, stained for Annexin V, and analyzed by flow cytometry.

Glycerol release assay. Adipocytes were subsequently washed two times with DMEM without phenol red (prewarmed to 37 °C). To initiate lipolysis, adipocytes were incubated with DMEM (without phenol red) containing 3% fatty acid-free BSA (Intergen, Purchase, NY) for 3 h. Released glycerol was expressed relative to the cellular protein content.

Statistics. Statistical analysis—data are presented as means and standard deviations (SD) or standard errors (SEM). Results were evaluated by one-way ANOVA and 2-tailed *t* test. Post hoc testing was carried out using the Tukey–Kramer multiple comparisons procedure.

Results

AICAR triggers dose-dependent apoptotic cell death in F442a adipocyte cells

To study the effect of AMPK activation on adipocyte apoptotic index, we initially evaluated the viability of F442a adipocyte cells in response to AICAR treatment. Cells were differentiated (as described under Materials and methods) and treated with increasing doses of AICAR. Cell viability was evaluated after 48 h of incubation by Annexin V staining and showed a significant increase in apoptotic index (Fig. 1A). Induction of apoptosis in adipocytes by AICAR was confirmed by immunoblot analysis of PARP cleavage in extracts from cells treated with the same doses of AICAR, (Fig. 1B). We then tested whether induction of apoptosis by AICAR in adipocytes was associated with AMPK activation in these cells. Immunoblot analysis of extracts from cells treated with AICAR for 1 h revealed a dose-dependent phosphorylation of the enzyme on Thr-172 (Fig. 1C), providing evidence for increasing AMPK activity in response to AICAR.

AICAR induces eIF2α phosphorylation

eIF2α is a regulator of protein inhibition in response to metabolic stress as after amino acid and glucose deprivation which have been demonstrated to activate AMPK [23]. To investigate the possible involvement of eIF2α in mediating some of the effects of AMPK in adipocyte, we evaluated the consequences of AICAR on eIF2α phosphorylation. Immunoblot analysis of extracts from cells treated with AICAR for up to 6 h demonstrated a significant elevation in the phosphorylated form of the α-subunit of the eIF2 (Fig. 2), demonstrating that AMPK activation led to inhibition of protein synthesis.

2-AP inhibition of eIF2α phosphorylation overcomes the apoptotic effects of AICAR

To further investigate whether eIF2α-induced translation inhibition could contribute to apoptotic cell death following AICAR treatment, we incubated the F442a

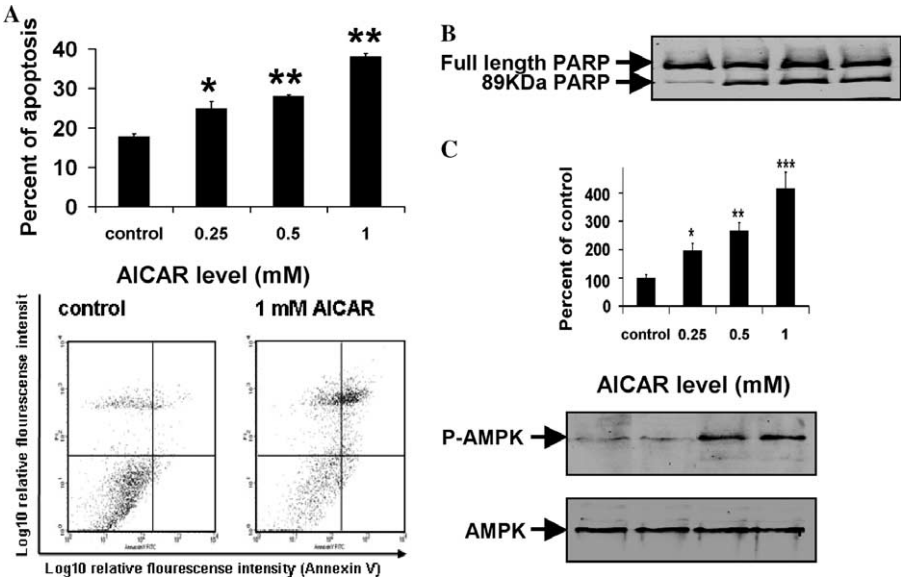

Fig. 1. Pro-apoptotic activity of AICAR on F442a adipocyte cells. (A) Differentiated F442a adipocytes were treated with 0–1 mM AICAR for 48 h. The cells were co-stained with Annexin V antibodies and propidium iodide, and then analyzed by flow cytometry. Apoptotic cells are represented by the two right-hand rectangles in each panel. *$p < 0.05$; **$p < 0.01$ vs. control following 2-tailed t test. (B) Differentiated F442a adipocytes were treated with 0–1 mM AICAR for 24 h and PARP cleavage was analyzed by Western blot. (C) Differentiated F442a adipocytes were treated with 0–1 mM AICAR for 1 h. AMPK activity was measured by Western blot analysis of AMPK expression and its phosphorylation. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ vs. control following 2-tailed t test.

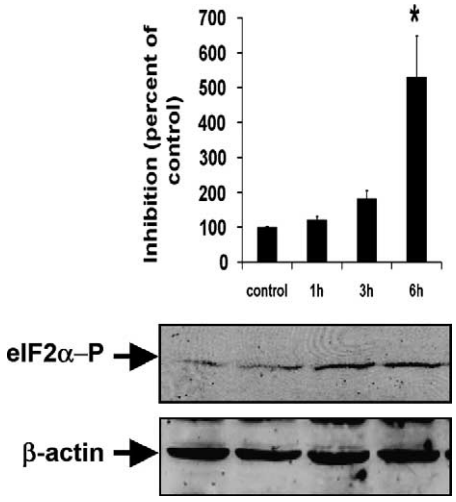

Fig. 2. AICAR induces eIF2α phosphorylation. Differentiated F442a adipocytes were treated with 1 mM AICAR for up to 6 h. EIF2α activity was measured by Western blot analysis of eIF2α expression and phosphorylation. *$p < 0.05$ vs. control following 2-tailed t test.

adipocytes with an increasing dose of AICAR together with 2-AP, a specific inhibitor of eIF2α phosphorylation. We noted a significant reduction of the apoptotic rate following 48 h of AICAR treatment according to Annexin V staining (Fig. 3A). Abrogation of adipocytes apoptosis following AICAR treatment was also confirmed by immunoblot analysis of PARP cleavage in extracts from cells treated with the same doses of AICAR (Fig. 3B).

2-AP inhibition of eIF2α phosphorylation overcomes reduction of adipogenic markers by AICAR

AICAR blocks the expression of late adipogenic markers and transcription factors essential for the adipogenic process [24]. To study the involvement of eIF2α in these effects, we evaluated the reduction of PPARγ and C/EBPα following AICAR treatment with or without the presence of 2-AP. While treatment of adipocytes with AICAR suppressed the protein level of these factors, supplementation of 2-AP could overcome protein reduction (Fig. 4).

2-AP inhibition of eIF2α phosphorylation overcomes lipolysis inhibition by AICAR

We next studied the involvement of eIF2α in the effect of AICAR on lipolysis, the breakdown of triglyceride to free fatty acid and glycerol. To this end, we used a concentration of 1 mM AICAR which provided us the highest degree of AMPK phosphorylation. Adipocytes were treated for different times 2–24 h with AICAR. AICAR treatment exhibits a reduction in the release of glycerol after 2 h of incubation reaching a significant inhibition after 12–24 h of treatment. Supplementation of 2-AP to the AICAR treated adipocytes overcame the anti-lipolytic effect (Fig. 5).

Discussion

In this study, we have investigated the metabolic and cellular effects of AMPK activation in F442a adipocytes. Previous research has focused on the involvement of

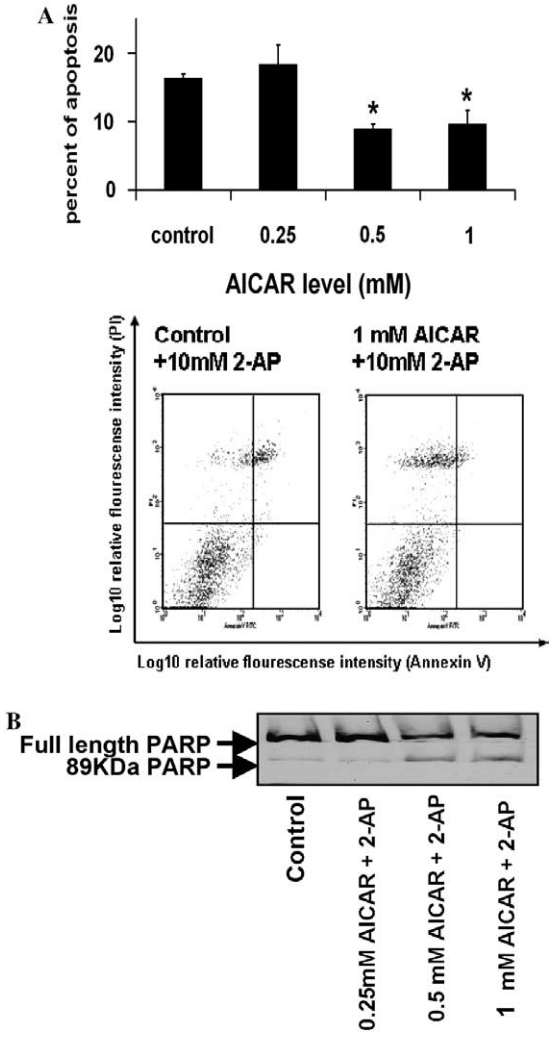

Fig. 3. Anti-apoptotic activity of AICAR supplemented with 2-AP on adipocyte cells. (A) Differentiated F442a adipocytes were treated with 0–1 mM AICAR and 5 mM 2-AP for 48 h. The cells were co-stained with Annexin V antibodies and propidium iodide, and were then analyzed by flow-cytometry. Apoptotic cells are represented by the two right-hand rectangles in each panel. *$p < 0.05$ vs. control following 2-tailed t test. (B) Cells were treated with 0–1 mM AICAR and 5 mM 2-AP for 24 h and PARP cleavage was analyzed by Western blot.

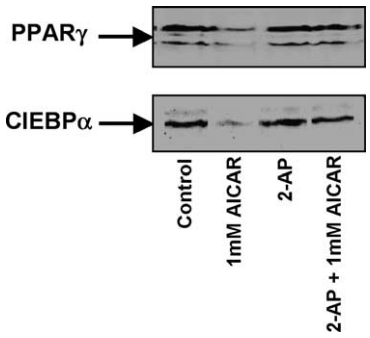

Fig. 4. Recovery of adipogenic marker expression by 5 mM 2-AP in 1 mM AICAR treated adipocyte cells. Differentiated 3T3-F442A cells were treated with 1 mM AICAR for 24 h. Expression levels of C/EBPa and PPARγ were analyzed by Western blot.

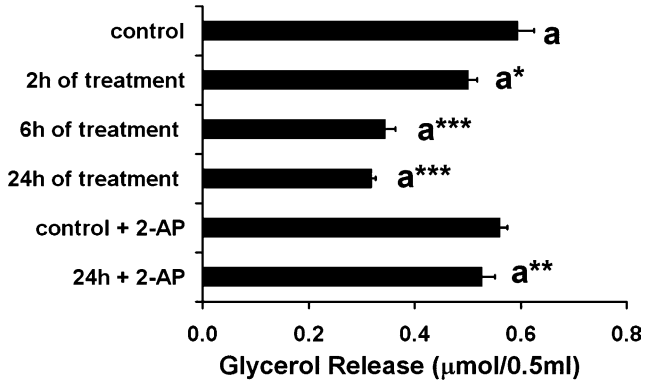

Fig. 5. Abrogation of anti-lipolytic effect of AICAR by 5 mM 2-AP. Differentiated F442a adipocytes were pre-incubated with serum free medium for 3 h + 2% fatty-acid free bovine serum albumin and incubated in the presence or the absent of 1 mM AICAR for 2–24 h with or without 5 mM 2-AP. a* $p < 0.05$; a** $p < 0.01$; a*** $p < 0.001$ vs. control following 2-tailed t test.

AMPK in triglyceride breakdown, with most reports indicating that AMPK antagonizes lipolysis. Our early experiments demonstrated the potential of AMPK to inhibit basal and induced lipolysis in different conditions (data not shown). However, environmental circumstances which lead to AMPK activation such as nutritional deprivation, adipokine stimulators, and pharmacological compounds which activate AMPK in adipose all result in fat depletion and not fat accumulation [25]. Thus far, we questioned whether AMPK may play a role in adipose regression through control of adipocyte cell number. Our results show that apoptosis of adipocytes could be induced progressively in response to AMPK activation by AICAR, supporting the assumption that the observed effects are targeted through AMPK. The main claim against the usage of AICAR to stimulate AMPK is its potential nonspecificity. Yet, this pharmacological activator enabled a dose-dependent increase in AMPK with different physiological outcomes depending on the magnitude of the stimulation. While up to a twofold increase in the phosphorylated form of AMPK was needed to produce a significant anti-lipolytic response, only higher degrees of phosphorylation produced a significant apoptotic effect. Say something as to what ''regulates'' this change. This should be taken into consideration when genetic approaches are used to activate AMPK (such as expression of the activated catalytic unit).

The next question addressed was to what extent are F442a adipocytes representative of the adipose tissue in vivo. In this regard, it is relevant that AICAR did not induce apoptotic effects in other in vitro models for peripheral activation of AMPK, such as HepG2 hepatocytes or C2C12 muscle cells, thus, suggesting the specificity of the cellular effect to adipogenic cells (unpublished results).

The involvement of AMPK in adipocyte apoptosis matches well the effects of leptin which activates adipose AMPK and is considered to produce an anti-obesity effect also via apoptotic cell death of adipocytes. Another exam-

ple is the anti-apoptotic effect of insulin in adipocytes parallel to its inhibition of AMPK activity [26].

Nutritional stress induces a program of coordinated gene expression designed to alleviate the underlying cellular disturbance or, alternatively induces apoptosis. In our previous studies, we have demonstrated the role of AMPK in the regulation of in vivo brain function in response to modest nutritional stress, while triggering apoptosis after severe nutritional deprivation [27]. The impact of nutrients on cellular gene expression is also very relevant to adipocyte function. eIF2α phosphorylation is part of a metabolic stress response to limited energy resources following nutrient deprivation [23,28]. In this sense, the ability of AMPK (which is considered to be a ''fuel gauge'' for cellular metabolism) to phosphorylate eIF2α fits makes ''biological sense'' and provides a novel pathway through which AMPK may modulate gene expression. Phosphorylation of eiF2α has a major impact on cell fate, since inhibition of the synthesis of cellular survival proteins may be one of the mechanisms responsible for metabolic stress apoptosis [29]. This may provide explaination for the apoptotic effect of AMPK activation observed in F442a adipocytes.

Finally, leptin and nutritional restriction negatively regulates adipose tissue content in part via down regulation of nuclear hormone receptor protein families C\EBP α, β, and δ [30]. These transcription factors are responsible for adipocyte-characteristic genes such as lipoprotein lipase, leptin, adipocyte fatty acid binding protein, and fatty acid synthase [24]. Further, previous studies demonstrated the involvement of eIF2α in the translation control of C/EBPα and C/EBPβ expression and their importance in cell viability. [31] Thus, our results, regarding the role of eIF2α in control of the adipogenesis transcription factors PPARγ and C/EBPα, may provide an additional mechanism through which energy balance may regulate adipose tissue mass.

Therefore, we speculate that AMPK may be the mediator for hormonal and nutritional effects on adipose tissue by specific translation down-regulation of central transcription factors like C/EBPα, PPARγ, and survival protein. It remains to be seen whether pharmacological manipulation of these pathways has any place in the therapeutic management of obesity.

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