The AMP-activated protein kinase prevents ceramide synthesis de novo and apoptosis in astrocytes

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Abstract Fatty acids induce apoptosis in primary astrocytes by enhancing ceramide synthesis de novo. The possible role of the AMP-activated protein kinase (AMPK) in the control of apoptosis was studied in this model. Long-term stimulation of AMPK with 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) prevented apoptosis. AICAR blunted fatty acid-mediated induction of serine palmitoyltransferase and ceramide synthesis de novo, without affecting fatty acid synthesis and oxidation. Prevention of ceramide accumulation by AICAR led to a concomitant blockade of the Raf-1/extracellular signal-regulated kinase cascade, which selectively mediates fatty acid-induced apoptosis. Data indicate that AMPK may protect cells from apoptosis induced by stress stimuli.

Key words: AMP-activated protein kinase; Apoptosis; Ceramide; Extracellular signal-regulated kinase; Astrocyte

1. Introduction

Mammalian AMP-activated protein kinase (AMPK) belongs to a family of protein kinases that has been highly conserved throughout evolution in animals, plants and yeast, and which plays a major role in cell response to metabolic stress [1,2]. AMPK is activated by AMP and by phosphorylation by an upstream kinase, which is itself activated by AMP. Once activated, AMPK phosphorylates and inactivates a number of regulatory enzymes involved in biosynthetic pathways. The AMPK cascade seems to have evolved to monitor the energy status of the cell and to initiate appropiate energy-conserving mechanisms in response to ATP depletion during metabolic stress [1,2]. One of the most intriguing and unexplored actions of AMPK is its possible anti-apoptotic effect. Thus, two previous reports have shown that pharmacological activation of AMPK protects thymocytes from dexamethasone-induced apoptosis [3] and Rat-1 fibroblasts from serum withdrawal-induced apoptosis [4]. However, the potential pathophysiological implications of these findings are hampered by the absolute lack of knowledge of the underlying mechanisms.

The present work was therefore undertaken to study the mechanism of the anti-apoptotic effect of AMPK in neural cells. One of the most important mediators of apoptosis in the central nervous system is ceramide. Thus, ceramide accumulation occurs in cultured neural cells exposed to stress stimuli, as well as in the brain in neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, epilepsy and ischemia/stroke [5,6]. Moreover, changes in ceramide metabolism exert important regulatory effects on neuronal growth and development [7]. We have recently developed a model in which exposure of primary astrocytes to fatty acids leads to apoptosis selectively via de novo-synthesized ceramide [8]. By using this model we sought to answer two questions: (a) does AMPK activation prevent ceramide-induced apoptosis?; (b) if so, which may be the targets of AMPK action?

2. Materials and methods

2.1. Cell culture

Cortical astrocytes were derived from 24 h old Wistar rats and cultured as described before [9]. For all the experimental determinations performed, the serum-containing medium was removed and cells were transferred to a chemically defined, serum-free medium consisting of DMEM/Ham's F12 (1:1, v/v) supplemented with 5 μg/ml insulin, 50 μg/ml human transferrin, 20 nM progesterone, 50 μM putrescine, 30 nM sodium selenite and 1.0% (w/v) defatted and dialyzed bovine serum albumin.

2.2. Cell death

Cell viability was determined by trypan blue exclusion. For Hoechst 33258 staining, cells were grown in glass coverslips and fixed (20 min at room temperature) with 4% paraformaldehyde in phosphate-buffered saline supplemented with 5% sucrose. The dye was applied at a final concentration of 16 μg/ml and cells were examined by fluorescence microscopy.

2.3. Rates of metabolic pathways

(a) Sphingolipid synthesis: reactions were started by the addition of 1 μCi L-[U-^{14}C]serine, and stopped with 1 ml methanol at the times indicated. Lipids were extracted and saponified, and ceramide and sphingomyelin were resolved by thin layer chromatography [8,10]. (b) Fatty acid oxidation: reactions were started by the addition of 0.2 mM (0.3 μCi) albumin-bound [1-^{14}C]palmitate plus 0.5 mM L-carnitine, and stopped with 0.3 ml 2 M $HClO_4$ after 2 h. Oxidation products were extracted and quantified exactly as described before [9]. (c) Fatty acid synthesis: reactions were started by the addition of 4 mM (1.0 μCi) [1-^{14}C]acetate, and stopped with 0.2 ml 10 M NaOH after 6 h. Samples were saponified and fatty acids were extracted with light petroleum ether [11].

2.4. Protein kinase activities

(a) AMPK: cells were lysed, supernatants were obtained, and

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Abbreviations: ACC, acetyl-CoA carboxylase; AICAR, 5-aminoimidazole-4-carboxamide ribonucleoside; AMPK, AMP-activated protein kinase; CPT-I, carnitine palmitoyltransferase I; ERK, extracellular signal-regulated kinase; SPT, serine palmitoyltransferase

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AMPK activity was determined as the incorporation of [γ-^{32}P]ATP into the specific SAMS peptide substrate [11]. (b) Extracellular signal-regulated kinase (ERK): cells were lysed, supernatants were obtained, and ERK activity was determined as the incorporation of [γ-^{32}P]ATP into a specific peptide substrate [10]. (c) Raf-1: after Raf-1 immunoprecipitation, Raf-1 kinase activity was determined as the incorporation of [γ-^{32}P]ATP into kinase-negative MEK1(97A) [10].

2.5. Other enzyme activities and levels

(a) Serine palmitoyltransferase (SPT): enzyme activity was determined in digitonin-permeabilized astrocytes as the incorporation of radiolabelled L-serine into ketosphinganine by a new procedure. Thus, the medium was aspirated and cells were washed twice with phosphate-buffered saline. Reactions were started by the addition of 100 mM HEPES, pH 8.3, 200 mM sucrose, 2.5 mM EDTA, 5 mM dithioerythritol, 50 μM pyridoxal phosphate, 1.0 mg/ml defatted and dialyzed bovine serum albumin, 15 μg/ml digitonin, 0.3 mM palmitoyl-CoA and 0.25 mM L-[U-^{14}C]serine (3 μCi/assay). After 45 min, reactions were stopped with 0.5 M NH_4OH and [^{14}C]ketosphinganine product was extracted with chloroform/methanol/1% NaCl [12]. Preliminary experiments defined the optimal concentration of palmitoyl-CoA, serine and digitonin in the assay, as well as its linearity with time. The validity of the assay was proved by the full inhibitory effect exerted by the SPT competitive inhibitor L-cycloserine. Western blot analysis was carried out with a polyclonal antibody raised against hamster SPT LCB2 catalytic subunit [13]. (b) Carnitine palmitoyltransferase I (CPT-I): enzyme activity was determined in digitonin-permeabilized astrocytes as the tetradecylglycidate-sensitive incorporation of L-[*Me*-^{3}H]carnitine into palmitoylcarnitine by method A ('one-step assay') described in [11]. Western blot analysis was carried out with a polyclonal antibody raised against rat liver CPT-I [9]. (c) Acetyl-CoA carboxylase (ACC): enzyme activity was determined in digitonin-permeabilized astrocytes as the incorporation of [1-^{14}C]-acetyl-CoA into fatty acids in a reaction coupled to the fatty acid synthase reaction [9]. Mass measurement of ACC was performed by avidin-based enzyme-linked immunosorbent assay analysis using a polyclonal antibody raised against rat liver ACC [14].

2.6. Statistical analysis

Results shown represent the means ± S.D. of the number of experiments indicated in every case. Statistical analysis was performed by ANOVA. A post hoc analysis was made by the Student–Neuman–Keuls test.

3. Results

3.1. Long-term AMPK stimulation prevents fatty acid-induced apoptosis

We have previously shown that exposure of primary astrocytes to palmitate leads to apoptosis [8]. Astrocytes were exposed to palmitate at a concentration physiologically relevant in brain, and cell viability was determined at different times. As shown in Fig. 1, palmitate induced the death of astrocytes in a time-dependent fashion. Prolonged exposure to 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), a selective cell-permeable activator of AMPK which has been widely used to demonstrate the implication of this kinase in the regulation of cellular processes [1], was able to prevent completely palmitate-induced cell death, as determined by both trypan blue exclusion (Fig. 1A) and Hoechst 33258 staining (Fig. 1B). In our cultured astrocyte system, incubation with 0.2 mM AICAR for 48 h activated AMPK by 47 ± 14% ($n = 3$, $P < 0.01$ vs. incubations with no additions).

3.2. Long-term AMPK stimulation prevents ceramide synthesis de novo and SPT induction

Fatty acid-induced apoptosis of astrocytes occurs selectively by enhanced ceramide synthesis de novo [8]. As shown in Table 1, palmitate notably stimulated ceramide synthesis de

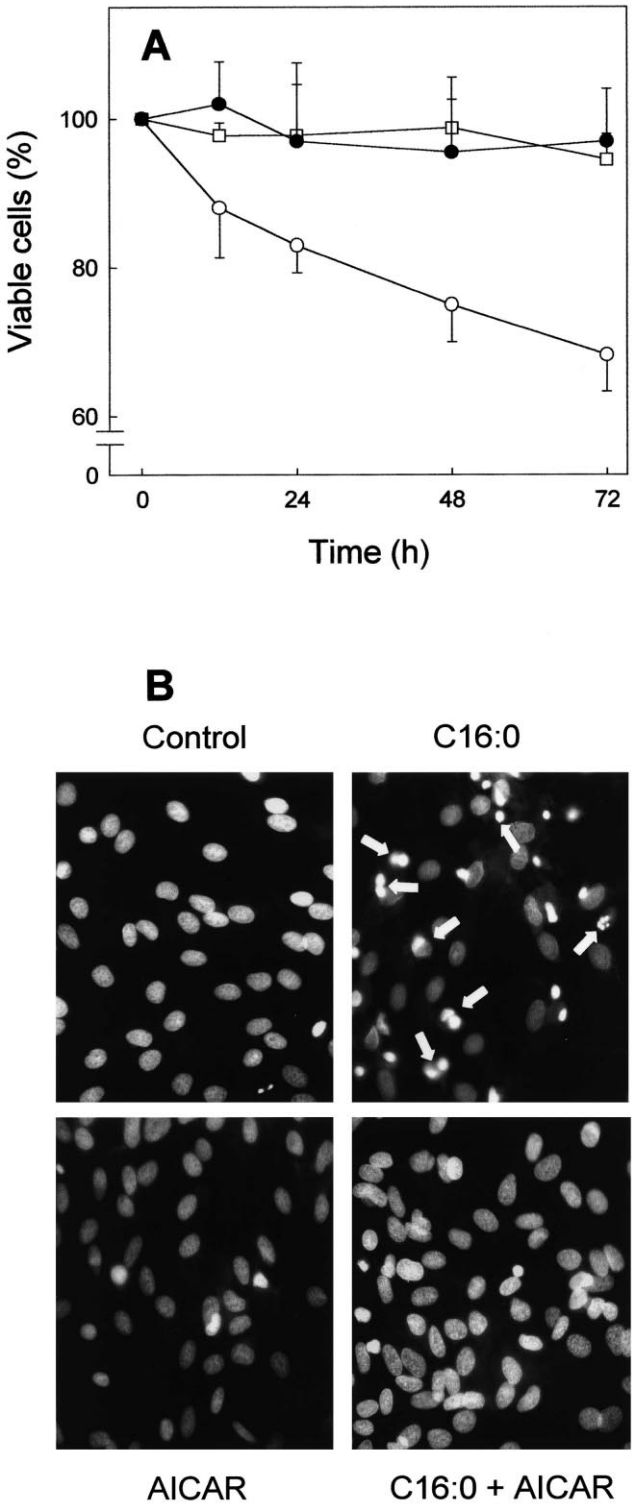

Fig. 1. Palmitate-induced death of astrocytes: prevention by AICAR. (A) Cell viability. Cells were incubated for the times indicated with 0.2 mM palmitate in the absence (○) or presence (●) of 0.2 mM AICAR, or with 0.2 mM AICAR alone (□). Results are expressed as percentage of incubations with no additions, and were obtained from four different experiments. (B) Hoechst 33258 staining. Cells were incubated for 48 h in the absence or presence of 0.2 mM palmitate (C16:0) and/or 0.2 mM AICAR. A representative experiment is shown. Similar results were obtained in two other experiments. Arrows point to condensed or fragmented nuclei.

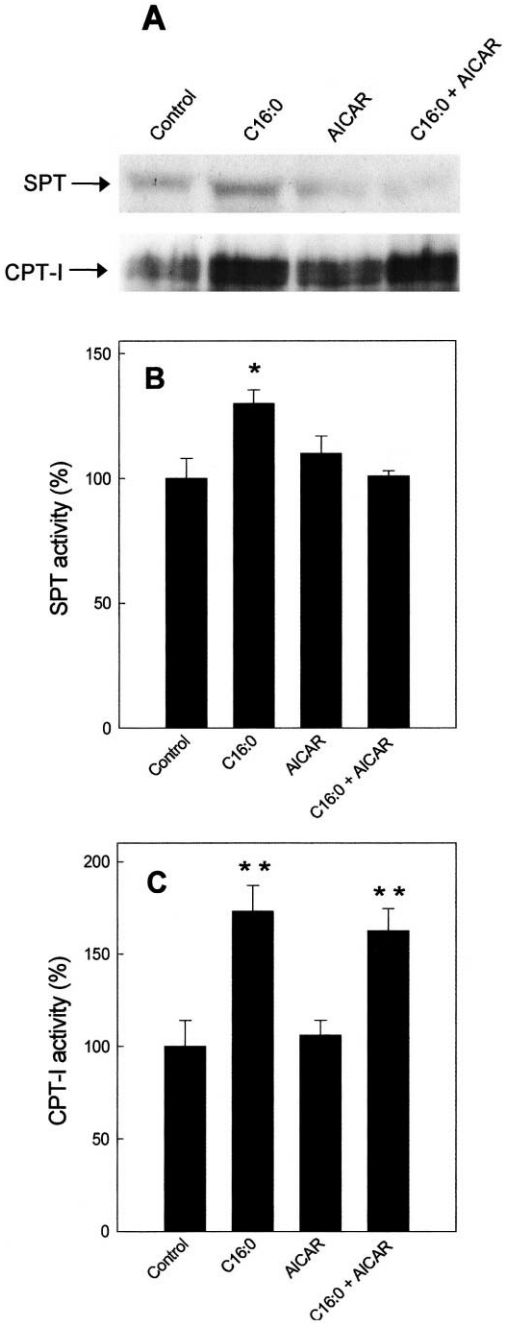

Fig. 2. Palmitate-mediated induction of SPT and CPT-I: differential effect of AICAR. Cells were incubated for 48 h in the absence or presence of 0.2 mM palmitate (C16:0) and/or 0.2 mM AICAR. (A) SPT and CPT-I expression. A representative Western blot is shown. (B) SPT activity. (C) CPT-I activity. Results in (B) and (C) are expressed as percentage of incubations with no additions, and were obtained from four different experiments. Significantly different from incubations with no additions: $*P < 0.05$; $**P < 0.01$.
←

novo. By contrast, no significant effect of palmitate on serine incorporation into sphingomyelin was evident. AICAR blunted palmitate-induced ceramide generation without exerting any effect on sphingomyelin synthesis. To further support the involvement of AMPK in the control of ceramide synthesis de novo we determined the activity of SPT, which catalyzes the pace-setting step of this pathway. Thus, AICAR prevented palmitate-mediated SPT induction, as determined by both Western blot (Fig. 2A) and assay of enzyme activity (Fig. 2B). Relative levels of SPT expression, as determined by densitometric analysis of the bands in the Western blots, were 1.00 (no additions), 1.37 ± 0.14 (48 h exposure to 0.2 mM palmitate), 1.01 ± 0.09 (48 h exposure to 0.2 mM AICAR), and 0.91 ± 0.15 (48 h exposure to 0.2 mM palmitate and 0.2 mM AICAR) ($n = 4$).

3.3. Long-term AMPK stimulation prevents Raf-1/ERK activation

We have previously shown that de novo-synthesized ceramide signals apoptosis in primary astrocytes via activation of the Raf-1/ERK cascade [8]. The effect of AICAR on the activity of these two kinases was therefore determined. The palmitate-induced activation of ERK (Fig. 3A) and Raf-1 (Fig. 3B) was prevented by AICAR. Although AMPK has been shown to phosphorylate a Raf-1 peptide in vitro [15], incubation of astrocytes with AICAR alone did not affect basal ERK and Raf-1 activities.

3.4. Long-term AMPK stimulation does not affect fatty acid catabolism

AMPK stimulation enhances fatty acid oxidation in astrocytes [11] and other cells such as myocytes and hepatocytes [1,2]. It might therefore be argued that AICAR prevents ceramide accumulation by diverting palmitate into the oxidative pathway. However, this is not the case in our experimental system. Although rapid (minute range) AMPK stimulation with AICAR does enhance fatty acid oxidation and blunt fatty acid synthesis in astrocytes [11], these alterations return to basal levels after prolonged (day range) AICAR challenge.

Table 1
Palmitate-induced ceramide synthesis and astrocyte death: prevention by AICAR

Additions	Viable cells (%)	L-[^{14}C]serine into lipid	
		Ceramide (%)	Sphingomyelin (%)
None	100 ± 8	100 ± 18	100 ± 14
C16:0	72 ± 7[a]	217 ± 36[a]	109 ± 25
AICAR	101 ± 9	93 ± 21	94 ± 8
C16:0+AICAR	95 ± 8	127 ± 34	91 ± 15

Cells were incubated for 48 h in the absence or presence of 0.2 mM palmitate (C16:0) and/or 0.2 mM AICAR. Results are expressed as percentage of incubations with no additions, and were obtained from six different experiments. 100% values of L-[^{14}C]serine incorporation into ceramide and sphingomyelin were 0.77 and 3.06 nmol per 24 h per mg cell protein, respectively.
[a]Significantly different ($P < 0.01$) from the respective incubations with no additions.

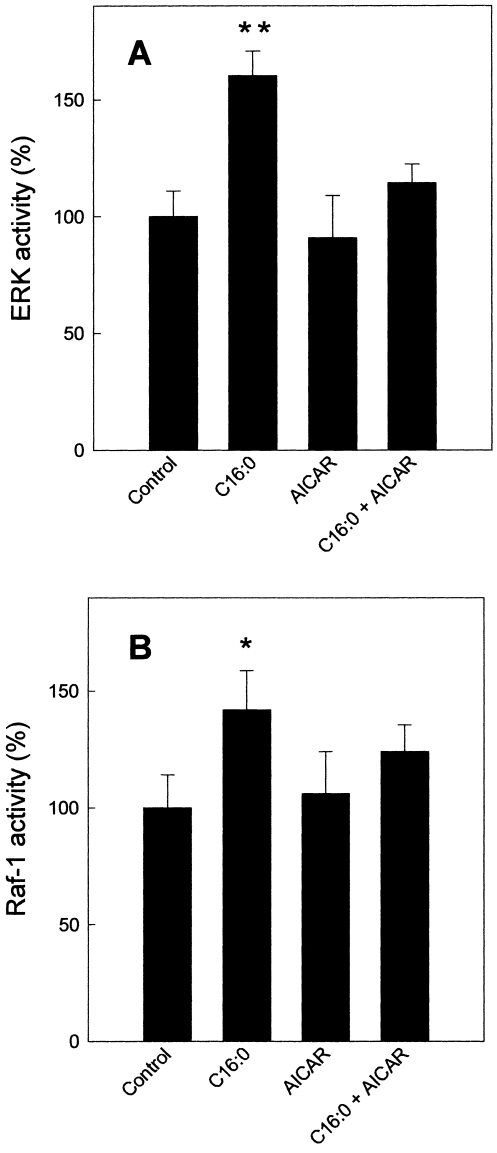

Fig. 3. Palmitate-induced activation of ERK and Raf-1 in astrocytes: prevention by AICAR. Cells were incubated for 48 h in the absence or presence of 0.2 mM palmitate (C16:0) and/or 0.2 mM AICAR. Results are expressed as percentage of incubations with no additions, and were obtained from four different experiments. (A) ERK activity. (B) Raf-1 activity. Significantly different from incubations with no additions: $*P < 0.05$; $**P < 0.01$.

Thus, no significant effect of 48 h AICAR exposure was evident on the rate of fatty acid oxidation and fatty acid synthesis (data not shown), and on the activity and levels of the pace-setting enzymes of these two pathways, CPT-I (Fig. 2A,C) and ACC (data not shown), respectively. Relative levels of CPT-I expression, as determined by densitometric analysis of the bands in the Western blots, were 1.00 (no additions), 1.61 ± 0.20 (48 h exposure to 0.2 mM palmitate), 1.05 ± 0.14 (48 h exposure to 0.2 mM AICAR), and 1.64 ± 0.11 (48 h exposure to 0.2 mM palmitate and 0.2 mM AICAR) ($n = 4$).

4. Discussion

The hypothesis that ceramide acts as a second messenger in the induction of apoptosis has attracted a lot of attention during the last few years. It is usually considered that ceramide generation through sphingomyelin hydrolysis by neutral and/or acid sphingomyelinase is the norm in ceramide signaling pathways leading to apoptosis. The link between receptor activation, sphingomyelinase stimulation and ceramide generation is mostly supported by comprehensive studies on the p55 tumor necrosis factor receptor, the p75 neurotrophin receptor and CD95/Fas [16,17]. In addition, other stress stimuli of chemical, physical, bacterial and viral origin may induce apoptosis by evoking changes in the sphingomyelin/ceramide cycle [16,17]. However, the de novo synthesis pathway has been gaining appreciation as an alternative means of generating an apoptotic pool of ceramide. Thus, a significant contribution of the de novo pathway to ceramide generation and apoptosis has been reported in a number of paradigms employing cultured neural (e.g. [8,10,18]) and non-neural cells (e.g. [19–21]). In particular, because palmitate is a precursor for ceramide synthesis de novo, it is conceivable that its accumulation may lead to increased ceramide synthesis and apoptosis, as evidenced in hematopoietic cells [22], pancreatic β cells [23] and astrocytes [8].

Work by Bazan in the early 70s demonstrated an enhanced breakdown of cellular glycerolipids and a concomitant accumulation of non-esterified fatty acids – including palmitic acid – in a number of models of brain trauma/ischemia [24,25]. The breakdown of membrane phospholipids on trauma/ischemia seems to be the result of Ca^{2+}-induced stimulation of phospholipases and of impairment of phospholipid resynthesis owing to energy depletion, and may be involved in irreversible damage of membrane structure and function [26]. In addition, non-esterified fatty acids exert various detrimental effects on brain structure and function such as uncoupling of oxidative phosphorylation; disruption of plasma membrane and mitochondrial ion fluxes; inhibition of membrane receptors, enzymes and ion channels; and elevation of synaptic glutamate concentration [26]. Hence our data indicate that ceramide synthesized de novo from non-esterified fatty acids may contribute to the apoptotic death of astrocytes in situations of brain trauma/ischemia. AMPK is expressed in brain [27–29], but its function in this organ is as yet unknown. Because in astrocytes (a) AMPK is stimulated in hypoxic [11] and gliotic states [30], and (b) AMPK activation prevents fatty acid-evoked SPT induction, stimulation of ceramide synthesis de novo and apoptosis (the present report), data also suggest that the balance between pro-apoptotic fatty acid accumulation and anti-apoptotic AMPK stimulation might determine ceramide levels and therefore influence the cell survival/death decision. In line with other studies showing that AMPK controls gene expression [31,32], cytoskeletal dynamics [33] and cell death [3,4], the present report supports the emerging notion that AMPK regulates not only energy metabolism but a much wider array of cellular functions.

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