FEBS Letters 526 (2002) 38–42 FEBS 26368

Sustained activation of AMP-activated protein kinase induces c-Jun N-terminal kinase activation and apoptosis in liver cells

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Received 12 July 2002; accepted 17 July 2002

First published online 26 July 2002

Edited by Jesus Avila

Abstract The aim of this work was to study the effect of a sustained activation of AMP-activated protein kinase (AMPK) on liver cell survival. AMPK activation was achieved by incubating FTO2B cells with AICA-riboside, which is transformed into ZMP, an AMP analogue, or by adenoviral transfection of hepatocytes with a constitutively active form of AMPK. Prolonged AMPK activation triggered apoptosis and activated c-Jun N-terminal kinase (JNK) and caspase-3. Experiments with iodotubercidin, dicoumarol and z-VAD-fmk, which inhibited AMPK, JNK and caspase activation, respectively, supported the notion that prolonged AMPK activation in liver cells induces apoptosis through an activation pathway that involves JNK and caspase-3.

Key words: AICA-riboside; AMP-activated protein kinase; Apoptosis; c-Jun N-terminal kinase; Hepatocyte

1. Introduction

The energy status of the cell, and especially ATP concentration, plays a crucial role in cell survival and prolonged ATP depletion leads to cell death. Small changes in ATP do not necessarily result in cell death but are sensed by cells. A fall in ATP with a subsequent rise in AMP activate the AMP-activated protein kinase (AMPK), which is considered as a 'fuel gauge' [1–3]. AMPK acts as a metabolic master switch by inactivating key enzymes of anabolic processes that consume ATP [2,4,5]. It also favors fatty acid oxidation [6] and, in certain cells, it stimulates glycolysis by increasing glucose transport and by activating 6-phosphofructo-2 kinase [7], thereby favoring ATP production. ATP conservation is therefore the net result of AMPK activation, which contributes to liver cell survival in the short term [8]. However, consequences of prolonged stress and of a sustained AMPK activation on cell survival have not been investigated. To study the effects of a sustained AMPK activation, cells were incubated with 5-aminoimidazole-4-carboxamide (AICA) riboside, an adenosine analogue, which activates AMPK after its phosphorylation to AICA-ribotide (ZMP), an AMP analogue [9]. This occurs in certain cells, such as hepatocytes or hepatoma cell lines [10,11]. Incubation of these cells with AICA-riboside also has the advantage of activating AMPK without affecting the adenine nucleotide levels [3], thus allowing the effects of prolonged AMP activation on cell survival to be studied independently of ATP depletion.

2. Materials and methods

2.1. Cell culture and adenoviral infection

FTO2B cells were maintained in Dulbecco's modified Eagle's medium/Ham's F12 medium (Life Technologies) supplemented with 10% fetal bovine serum (BioWhittaker Europe), 75 mg/l penicillin sulfate and 100 mg/l streptomycin. Rat hepatocytes were isolated and cultured as in [12]. Z-Val-Ala-Asp-fluoromethylketone (z-VAD-fmk, from Bachem), dicoumarol and iodotubercidin (from Sigma) were added 30 min before AICA-riboside (from Sigma). Primary cultures of hepatocytes were infected either with a control adenovirus (Ad-Null), or with an adenovirus containing the constitutively active form of AMPK (Ad-CA-AMPK) as in [13].

2.2. Detection and quantification of apoptosis

To demonstrate apoptosis, four methods were used: (1) the percentage of cells containing sub-G1 (apoptotic) nuclei were counted by FACS analysis [14,15]; (2) phosphatidylserine externalization was measured by labelling the cells with Annexin V-FITC (100 μg) (R&D Systems) and propidium iodide (0.1 μg), and monitored by FACS analysis (FITC, emission at 527 nm; propidium iodide, emission at 590 nm); (3) DNA laddering was detected after extraction of FTO2B cells DNA using the 'apoptotic DNA ladder kit' (from Amersham) and electrophoresis in a 2% agarose gel containing ethidium bromide; and (4) caspase-3 activity was assayed using Ac-DEVD-AMC (*N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin) as fluorogenic substrate (from Biosource). Cells were lysed in a buffer containing 0.1% (w/v) CHAPS, 10 mM dithiothreitol (DTT), 10 mM HEPES pH 7.4, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 20 μg/ml pepstatin. Cell lysates (25–50 μg of protein) were incubated with 50 μM Ac-DEVD-AMC for 3 h at 20°C and AMC fluorescence was measured (excitation at 360 nm, emission at 460 nm). The rate of AMC produced was linear as a function of time and of the amount of extracts. Caspase-3 activity is expressed as pmol of AMC formed/min/mg of protein.

2.3. Protein kinase assays and other methods

AMPK activity was measured as in [7]. The activity of c-*myc*-tagged CA-AMPK protein was measured as in [13]. c-Jun N-terminal kinase (JNK) activity was measured in cell lysates (300 μg of protein) after

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Abbreviations: AMPK, AMP-activated protein kinase; JNK, c-Jun N-terminal kinase; AICA-riboside, 5-aminoimidazole-4-carboxamide-riboside

0014-5793/02/$22.00
PII: S0014-5793(02)03110-1

immunoprecipitation with 300 ng of anti-JNK1 antibody (kindly provided by J. Vandenheede, KUL, Leuven, Belgium) bound to protein A-Sepharose (from Pharmacia Biotech). The immunoprecipitates were first washed in PBS containing 1 mM Na_3VO_4 and 1% (w/v) NP-40, and then in kinase reaction buffer (20 mM HEPES, pH 7.5, 2 mM EGTA, 25 mM $MgCl_2$, 1 mM DTT, 0.1% (w/v) Triton X-100, 1 mM Na_3VO_4), and incubated with 1 μg GST-c-jun-1–79 (Alexis) in the presence of 20 μM Mg[γ-^{32}P]ATP (27 500 cpm/pmol, Amersham). Phosphorylation of GST-c-jun-1–79 was quantified by phosphorimaging after 10% SDS–PAGE. One unit of protein kinase activity corresponds to 1 nmol of phosphate incorporated/min. Protein was estimated by Coomassie blue staining (Bio-Rad). Student's two-way *t*-test for paired data was used to assess the statistical significance of differences.

3. Results

3.1. AICA-riboside induces apoptosis in FTO2B cells

Incubation of FTO2B cells with 2 mM AICA-riboside led to AMPK activation, which was detectable after 15 min, maximal after 1 h and remained above the control values for at least 24 h (Fig. 1A). At 24 h, labelling the cells with propidium iodide indicated that the number of dead cells increased (data not shown). The occurrence of apoptosis was demonstrated as follows. First, the percentage of nuclei containing subdiploid (sub-G1) amounts of DNA in FTO2B cells incubated for 36 h with different concentrations of AICA-riboside increased from 13 ± 1.1 (controls) to 68 ± 1.5 (2 mM AICA-riboside, $n = 5$). Second, DNA laddering was observed after 24 h with 2 mM AICA-riboside (Fig. 2A). Third, a dose-dependent increase in the externalization of phosphatidylserine was detected by annexin V labelling (Fig. 3).

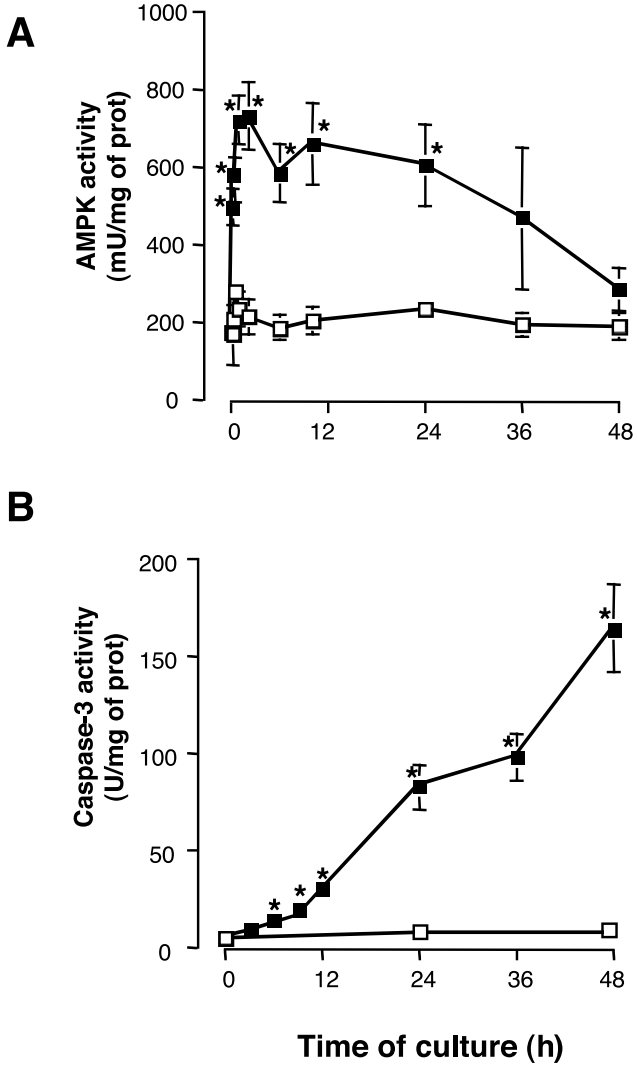

Fig. 1. Activation of AMPK (A) and caspase-3 (B) in FTO2B cells incubated with AICA-riboside. FTO2B cells were cultured for the indicated periods of time without (open squares) or with 2 mM AICA-riboside (filled squares). The values are the means ± S.E.M. for five (A) or six (B) experiments. *Significantly different from the control values ($P < 0.05$).

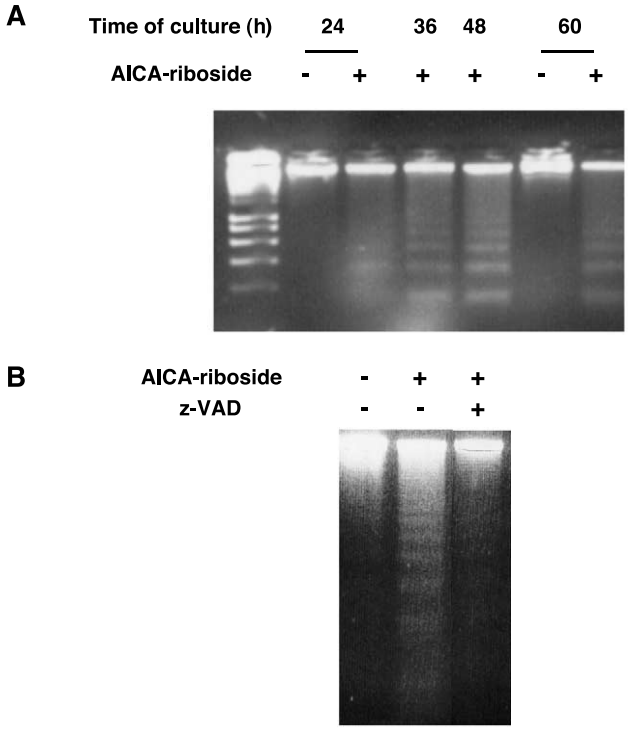

Fig. 2. DNA laddering in FTO2B cells incubated with AICA-riboside. A: FTO2B cells were cultured in the absence (−) or in the presence (+) of 2 mM AICA-riboside (AICAr) for the indicated periods of time. B: FTO2B cells were cultured in the absence (−) or in the presence (+) of 5 mM AICA-riboside (AICAr) for 48 h without (−) or with (+) 100 μM z-VAD-fmk (z-VAD).

DNA fragmentation and phosphatidylserine externalization were blocked by z-VAD-fmk, the general caspase inhibitor (Figs. 2B and 3). Accordingly, exposure to 2 mM AICA-riboside significantly increased the activity of caspase-3, the major effector caspase, after 6–8 h of treatment and onwards (Fig. 1B); the effect was apparent at 1 mM AICA-riboside and maximal at 5 mM AICA-riboside (data not shown). Caspase-3 activation was prevented if AICA-riboside was removed from the culture medium within the first 10 h of incubation. After this critical time point, apoptosis was inescapable (data not shown). Therefore, a sustained AICA-riboside treatment was required to induce apoptosis. The delay between AMPK activation and the onset of caspase-3 activation suggests that an intermediate signal mediates apoptosis induction.

3.2. AMPK activation leads to JNK activation and is required for the induction of apoptosis

Activation of stress-activated protein kinases, such as JNK

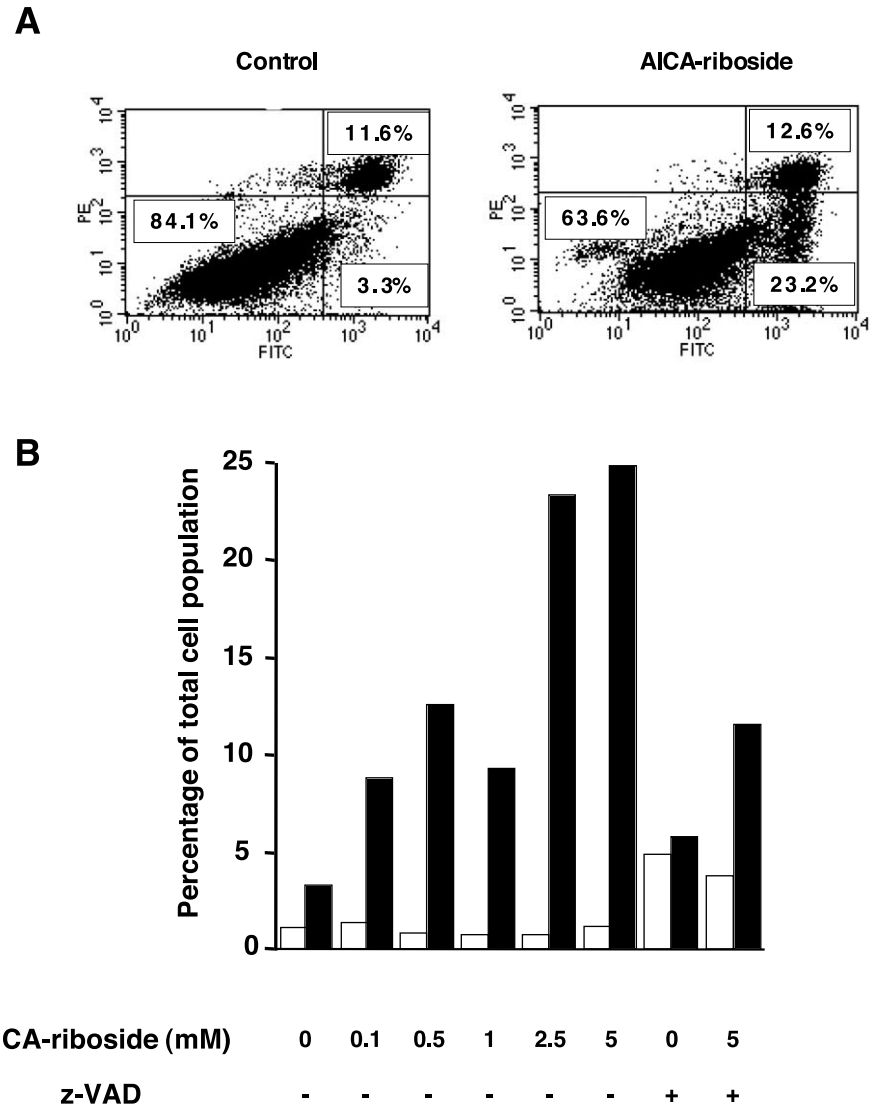

Fig. 3. FACS analysis of FTO2B cells labelled with annexin V and propidium iodide after incubation with AICA-riboside. A: FTO2B cells were cultured for 24 h in the absence (control) or in the presence of 2.5 mM of AICA-riboside. Cells were stained with annexin V and propidium iodide and analyzed by flow cytometry as described in Section 2. The *y*-axis represents the propidium iodide channel; the *x*-axis represents the annexin channel. Percentage of total cells in quadrants corresponds to living cells (propidium iodide- and annexin V-negative cells, left), necrotic and late apoptotic cells (propidium iodide- and annexin V-positive cells, upper right), and early apoptotic cells (annexin V-positive cells, lower right). B: FTO2B cells were cultured in the presence of the indicated concentrations of AICA-riboside (AICAr) for 24 h in the absence (−) or in the presence (+) of 100 μM of z-VAD-fmk and analyzed as in Fig. 3A. Black columns: annexin V-positive cells, white columns: propidium iodide-positive cells.

or p38 mitogen-activated protein kinase, induces apoptosis in certain cells [16,17]. No activation of p38 could be detected in FTO2B cells treated with AICA-riboside and SB203580, the inhibitor of p38 activation, did not prevent apoptosis (data not shown). By contrast, JNK activation was detectable within 1 h, was maximal at 2 h and persisted for at least 10 h after AICA-riboside exposure (Fig. 4A), indicating that JNK activation followed AMPK activation and preceded caspase-3 activation. Moreover, z-VAD-fmk did not prevent JNK activation by AICA-riboside (data not shown), suggesting that caspases did not mediate JNK activation.

Incubation of FTO2B cells with 10 μM iodotubercidin, an inhibitor of adenosine kinase which blocks ZMP formation from AICA-riboside [18], completely blocked the activation of AMPK, JNK and caspase-3 induced by AICA-riboside (data not shown). On the other hand, dicoumarol, an inhibitor of JNK activation [19], prevented the activation of both JNK and caspase-3 (Fig. 4B) but not AMPK (data not shown). These results suggest that AICA-riboside induces apoptosis through an activation cascade involving AMPK, JNK and subsequently caspase-3.

Finally, the pro-apoptotic effect of AICA-riboside was confirmed in primary rat hepatocytes, which indeed displayed an increase in the percentage of apoptotic nuclei as well as an activation of JNK and caspase-3 (data not shown). To demonstrate that AMPK activation by itself suffices to induce apoptosis, we infected hepatocytes with an adenovirus encoding a truncated α1-AMPK catalytic subunit (c-*myc* tagged, and with a Thr-172-Asp mutation in the activation loop). The mutated subunit acts as a constitutively active form of

AMPK when overexpressed in hepatocytes [13]. Expression of the constitutively active AMPK resulted in the activation of both JNK and caspase-3 (Fig. 5), thus demonstrating that AMPK activation is sufficient to induce apoptosis.

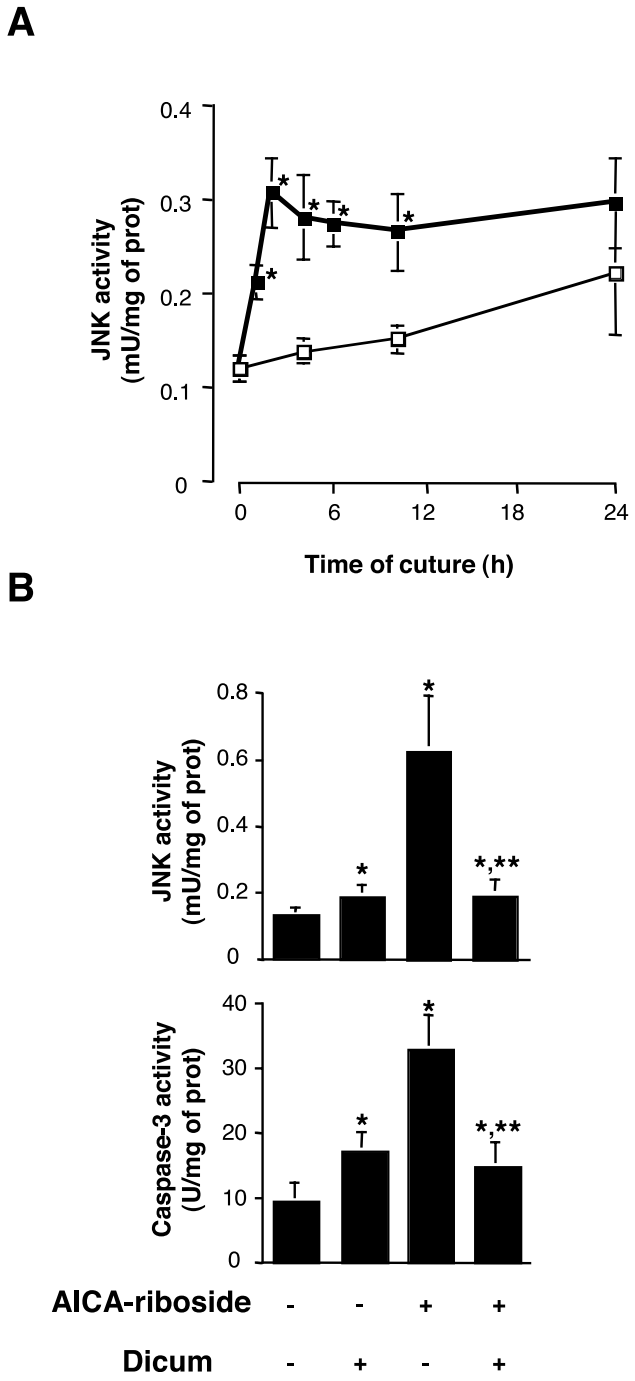

Fig. 4. Effects of AICA-riboside and dicoumarol on JNK and caspase-3 activities in FTO2B cells. A: FTO2B cells were cultured for the indicated periods of time without (open squares) or with 2 mM AICA-riboside (filled squares). B: Effects of pre-treatment with 100 μM of dicoumarol (Dicum) on JNK and caspase-3 activation in FTO2B cells exposed to 5 mM AICA-riboside for 1.5 and 15 h, respectively. The values are the means ± S.E.M. for five experiments. *Significantly different from the control values; **significantly different from the AICA-riboside-treated cells values ($P < 0.05$).

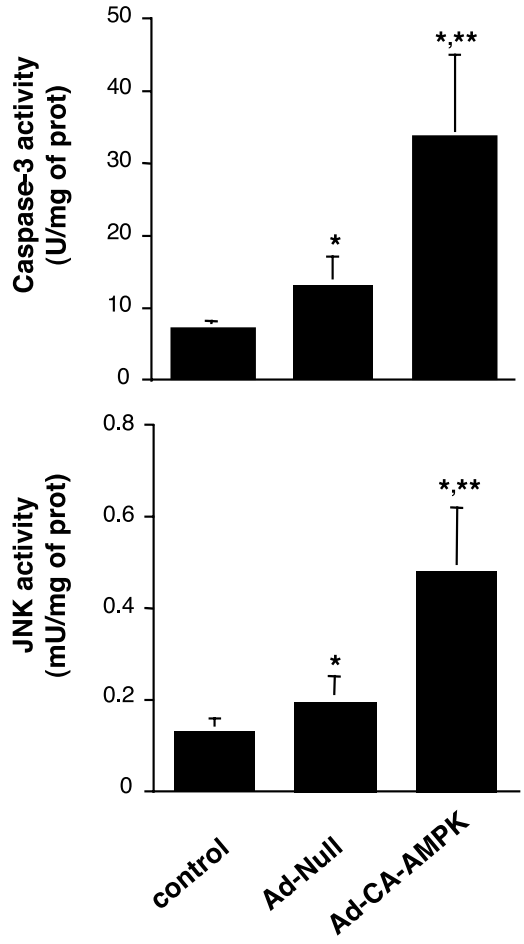

Fig. 5. Infection of hepatocytes with adenovirus containing the constitutively active form of AMPK induces JNK and caspase-3 activation. Primary rat hepatocytes were infected with control adenovirus (Ad-Null) or with adenovirus expressing the constitutively active form of AMPK (Ad-CA-AMPK) at 30 pfu/cell, or remained uninfected (control) for 30 h. Specific activities of JNK and caspase-3 were determined as described in Section 2. The values are the means ± S.E.M. for six independent experiments. *Significantly different from the control values; **significantly different from the Ad-Null-infected cells values ($P < 0.05$).

4. Discussion

Here we demonstrate that AMPK activation can lead to the induction of apoptosis in liver cells. This is the first demonstration of a pro-apoptotic effect of AMPK. Our results also suggest that apoptosis induction by AMPK is mediated by JNK. AMPK activation had to be sustained (> 10 h) to trigger apoptosis. This time course is in agreement with the previous observations that a sustained rather than a transient JNK activation is associated with apoptosis [20]. The delay between AMPK activation and the onset of apoptosis may correspond to the time required to accumulate pro-apoptotic proteins in excess of anti-apoptotic proteins. Proteins such as p53, Bax and Fas ligand have indeed been shown to be upregulated or activated by JNK [21–23].

Our results contrast with earlier reports which showed that AICA-riboside inhibits apoptosis induced by dexamethasone, fatty acids or high glucose concentrations in thymocytes, astrocytes and human umbilical vein endothelial cells, respectively [24–26]. In these experiments, AICA-riboside was antag-

onizing the pro-apoptotic effects of various substances. However, we have no simple explanation for the difference between these results and our data, but it is likely that the effect of AMPK activation on cell survival may depend on the cell type, the environmental conditions and on the duration of kinase activation.

Finally, we speculate that prolonged AMPK activation favors apoptosis over necrosis, because of the ATP-preserving effects of its activation. However, if ATP depletion is drastic, as is the case during anoxia, AMPK activation is transient [7] and necrosis rather than apoptosis is expected. Therefore it seems that the duration of AMPK activation, as well as the extent and rapidity of ATP depletion, might be crucial for determining the type of cell death.

Acknowledgements: We thank D. Carling (London), U. Krause, D. Vertommen, A.S. Marsin, L. Bertrand, S. Jindrichova, J.C. Renaud and B. Lauwerijs for their help and interest, Mrs. M. Couvreur and Mrs. V. O'Connor for technical and secretarial assistance. D.M. held an ICP-Michel De Visscher Fellowship. C.B. is supported by the Belgian Fund for Scientific Research. This work was supported by services of the Belgian Federal Programme Interuniversity Poles of Attraction (P5), the French Community of Belgium (ARC, 98/03-216), the Belgian Fund for Medical Scientific Research and the European Programme (QLG1-CT-2001-01488, AMPDIAMET).

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