5-Aminoimidazole-4-carboxamide riboside induces apoptosis in Jurkat cells, but the AMP-activated protein kinase is not involved

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5-Aminoimidazole-4-carboxamide (AICA) riboside, a precursor of purine nucleotide biosynthesis, induces apoptosis in Jurkat cells. Incorporation of AICAriboside into the cells is necessary for this effect since addition of nitrobenzylthioinosine, a nucleoside-transport inhibitor, completely protects Jurkat cells from apoptosis. Adenosine, but not other nucleosides, also protects Jurkat cells from AICAriboside-induced apoptosis. The apoptotic effect is caspase-dependent since caspases 9 and 3 are activated and the caspase inhibitor benzoyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD.fmk) blocks apoptosis. Furthermore, AICAriboside induces mitochondrial cytochrome c release. AICAriboside, when phosphorylated to AICAribotide (ZMP), is a specific activator of the AMP-activated protein kinase (AMPK) in certain cell types. However, AICAriboside does not activate AMPK in Jurkat cells. Moreover, 5-iodotubercidin, an inhibitor of AICAriboside phosphorylation, does not inhibit apoptosis in Jurkat cells. These results indicate that AICAriboside induces apoptosis independently of ZMP synthesis and AMPK activation in Jurkat cells.

Key words: 5-aminoimidazole-4-carboxamide (AICA) riboside, AMPK, apoptosis, caspases, nucleotides.

INTRODUCTION

The nucleoside 5-aminoimidazole-4-carboxamide riboside (AICAriboside), when phosphorylated to AICAribotide (ZMP), can enter the late steps of purine biosynthesis de novo. AICAriboside has various effects in several types of eukaryotic cells. These effects include inhibition of growth and depletion of pyrimidine nucleotide pools in Chinese hamster ovary fibroblasts [1,2], accelerated repletion of purine nucleotide pools in heart [3], reduction of endurance in skeletal muscle [4], inhibition of fatty acid, sterol synthesis and gluconeogenesis in hepatocytes [5–8], and an increase of glucose uptake in muscle [9,10]. When AICAriboside is phosphorylated to ZMP it mimics 5'-AMP and activates the AMP-activated protein kinase (AMPK) [6]. The effects of AICAriboside on glucose and lipid metabolism are mediated through activation of AMPK [6–10].

Recently, several authors have described AICAriboside as an inhibitor of apoptosis in different cell types. AICAriboside inhibits glucocorticoid-induced apoptosis in quiescent thymocytes [11], apoptosis caused by serum deprivation in fibroblasts overproducing fructose 2,6-bisphosphate [12], ceramide-induced apoptosis in astrocytes [13] and hyperglycaemia-induced apoptosis in endothelial cells [14]. Since AICAriboside has been used as a specific activator of AMPK [6], it has been suggested that the anti-apoptotic effects of AICAriboside are mediated through activation of AMPK.

However, a toxic effect of AICAriboside has been reported in different cell lines, including human B- and T-lymphoblasts [1,15–17]. These apparently contradictory results suggest that AICAriboside has toxic effects independent of AMPK activation or, alternatively, that AMPK can induce apoptosis in certain cell lines. In this paper we report that AICAriboside induces apoptosis in Jurkat cells after incorporation into the cell, and that this effect is caspase-dependent. We also show that AMPK does not mediate the apoptosis induced by AICAriboside. Indeed, phosphorylation of AICAriboside to ZMP is not necessary to induce apoptosis in Jurkat cells.

EXPERIMENTAL

Reagents

AICAriboside, guanosine, uridine, adenosine, cytidine, ZMP, ATP, ADP, AMP, GTP, GDP, GMP, propidium iodine (PI), 3,4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT), nitrobenzylthioinosine (NBTI) and oligomycin were from Sigma (St. Louis, MO, U.S.A.). Annexin V-FITC was from Bender MedSystems (Vienna, Austria). Z-VAD.fmk (benzoyloxycarbonyl-Val-Ala-Asp-fluoromethylketone) was from Bachem AG (Bubendorf, Switzerland). 5-Iodotubercidin was from Biomol Research Labs (Plymouth Meeting, PA, U.S.A.).

Cell culture

The human cell line Jurkat was from the European Collection of Cell Cultures. Cells were grown in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum, 1% glutamine and 1% penicillin/streptomycin at 37°C in a humidified atmosphere containing 5% CO₂.

Analysis of apoptosis by flow cytometry

Between 150000 and 300000 cells were washed in PBS and resuspended in 150 μl of annexin binding buffer and incubated...
with 0.4 µl of annexin V-FITC. After 20 min of incubation in the dark at room temperature, 150 µl of annexin binding buffer with 3 µl of PI (50 µg/ml) were added just before flow-cytometric analysis. Data were analysed using Cell Quest software (Becton Dickinson). Cell viability was measured as the percentage of the cell population that were annexin V- and PI-negative.

Western blot analysis

Whole-cell protein extracts were obtained by lysing cells with Laemmli sample buffer [18]. Protein concentration was measured with the Micro BCA Protein Assay Reagent Kit (Pierce, Rockford, IL, U.S.A.). The protein extract (50 µg) was subjected to SDS/PAGE and transferred to Immobilon-P membranes (Millipore, Bedford, MA, U.S.A.). Membranes were blocked for 1 h with 5% dried skimmed milk in TBST (50 mM Tris HCl, pH 8.0, 150 mM NaCl and 0.5% Tween-20) and then incubated with the following antibodies: polyclonal anti-phospho-AMPK (Thr-172; Cell Signalling Technology, Beverly, MA, U.S.A.), polyclonal anti-phospho-acetyl-CoA carboxylase (ACC) (Ser-79; Upstate, Lake Placid, NY, U.S.A.), polyclonal anti-poly(ADP-ribose) polymerase (PARP; Boehringer Mannheim), polyclonal anti-caspase-3 (Transduction Laboratories, Lexington, KY, U.S.A.), polyclonal anti-caspase-9 (New England Biolabs, Beverly, MA, U.S.A.), monoclonal anti-α-tubulin (Oncogene Science, Uniondale, NY, U.S.A.), or monoclonal anti-Bcl-2 (Dako A/S, Glostrup, Denmark). Antibody binding was detected with horseradish peroxidase-coupled secondary antibody and the enhanced chemiluminescence (ECL) detection kit (Amersham Biosciences, Little Chalfont, Bucks., U.K.).

Cytochrome c release measurements

Release of cytochrome c from mitochondria to cytosol was measured by Western blotting as described previously [19]. Mitochondrial and cytosolic fractions were lysed with sample buffer and electrophoresed on a 15% polyacrylamide gel and then analysed by Western blot using monoclonal antibodies against cytochrome c (Pharmingen, San Diego, CA, U.S.A.), cytochrome oxidase subunit II (Molecular Probes, Eugene, OR, U.S.A.) or mitogen-activated protein kinase (MAP kinase) 2 (Upstate Biotechnology, Lake Placid, NY, U.S.A.).

Nucleotide determination

For nucleotide analysis 10^7 cells were used. After incubation, the cells were harvested by centrifugation, washed twice in PBS and centrifuged again. The supernatant was removed, and the nucleotides were extracted from the cell pellet with 300 µl of ice-cold 10% perchloric acid. The extract was left on ice for 15 min, mixed and subsequently centrifuged at 12000 g for 5 min at 4 °C. The supernatant was separated from the pellet, neutralized with 5 M potassium carbonate and filtered with Ultrafree-MC Centrifugal Filter Units (nominal molecular-mass limit, 10000 Da; Millipore, Bedford, MA, U.S.A.) by centrifugation at 12000 g for 30 min at 4 °C. Neutralized extracts were frozen and stored at —80 °C for HPLC analysis. The protein content of the cells was determined in the pellet after perchloric acid extraction, according to the Micro BCA Protein Assay Reagent Kit. Nucleotide analyses were carried out as follows. A 20 µl sample was injected on to a Spherisorb 5 SAX anion-exchange column (2.5 cm x 4.6 mm) using a gradient of 5 mM NH₄H₂PO₄, pH 2.5 (buffer A), and 500 mM NH₄H₂PO₄, pH 3.9 (buffer B), at a flow rate of 1 ml/min. A linear gradient was developed over 40 min from 0% to 100%, buffer B. An LKB model 2152 HPLC equipped with a Kontron 432 detector was used. The various peaks in the extracts were identified by comparison of retention times with known external standards and relative absorbance at 260 nm. The following nucleotides were routinely quantified in the extracts: ATP, ADP, AMP, ZMP, GTP, GDP and GMP. The results are expressed as nmol/10^7 cells.

RESULTS

AICArriboside induces apoptosis in Jurkat cells

We studied the effect of several doses of AICArriboside, ranging from 2 to 8 mM, on the viability of Jurkat cells. AICArriboside at 2 mM did not induce apoptosis significantly after 24 h of incubation, measured by phosphatidylserine exposure. However, higher concentrations of the nucleoside induced apoptosis in a dose-dependent manner. A concentration of 4 mM AICArriboside reduced viability by 45%, in Jurkat cells (Figure 1A). Similar
AMP-activated protein kinase-independent apoptosis induced by AICAriboside

Adenosine and NBTI protect cells from AICAriboside-induced apoptosis

Results were obtained when viability was determined by MTT assay (results not shown). A time-course study was performed with 4 mM AICAriboside. A significant decrease in cell viability was seen after 6 h of incubation with AICAriboside (Figure 1B). This effect was greater when cells were incubated for 24 h.

Adenosine and NBTI protect cells from AICAriboside-induced apoptosis

It has been reported that AICAriboside produces pyrimidine nucleotide starvation in Chinese hamster fibroblasts and that addition of uridine completely reverses the toxicity produced by AICAriboside [1]. We studied the effect of the addition of different nucleosides to Jurkat cells, and 100 μM adenosine partially protected from AICAriboside-induced apoptosis (results not shown). This protection was complete with 2 mM adenosine (Figure 2A). However, uridine and other nucleosides, at the same concentration, did not protect Jurkat cells from apoptosis. We also analysed whether the apoptotic effect of AICAriboside was dependent on AICAriboside uptake. We found that NBTI, a nucleoside-transport inhibitor [20], completely inhibits AICAriboside-induced apoptosis at low doses.

Figure 2  Adenosine and NBTI protect cells from AICAriboside-induced apoptosis

Cells were incubated for 24 h with the combination of 4 mM AICAriboside and different nucleosides at 2 mM (A) or NBTI at different doses (B). Viability was measured by analysis of phosphatidylserine exposure and PI uptake as described in the Experimental section. Data are shown as means ± S.E.M. from two experiments in duplicate.

Figure 3  Apoptosis induced by AICAriboside in Jurkat cells is caspase-dependent and involves cytochrome c release

(A) Z-VAD.fmk protects cells from AICAriboside-induced apoptosis. Jurkat cells were preincubated for 1 h with 200 μM Z-VAD.fmk (black bars) or an equivalent amount of DMSO solvent as a control (white bars), after which 4 mM AICAriboside was added for 24 h. Viability was measured by analysis of phosphatidylserine exposure and PI uptake as described in the Experimental section. Data are shown as means ± S.E.M. from two experiments in duplicate. (B) Effect of AICAriboside on PARP cleavage and analysis of the processing of caspases 9 and 3. Cells were incubated with 4 mM AICAriboside for different times and whole-cell extracts were analysed by Western blot with antibodies against PARP, caspase-9 and caspase-3. The migration position of the native PARP (116 kDa) and the proteolytic fragment (85 kDa), the precursor form of caspase-3 (32 kDa) and the cleavage product of caspase-9 (37 kDa) are indicated. Bcl-2 (26 kDa) was also analysed. (C) Analysis of cytochrome c release. Jurkat cells were treated with 4 mM AICAriboside and mitochondrial and cytosolic extracts were analysed by Western blot at different times. MAP kinase 2 was analysed as a control for protein loading. Cytochrome oxidase II was analysed as a control of cytosolic fraction purification. Viability was measured by analysis of phosphatidylserine exposure and PI uptake as described in the Experimental section.
(50 nM; Figure 2B). This result indicates that the AICAriboside transporters belong to the NBTI-sensitive class (see Discussion) and, importantly, that AICAriboside uptake is necessary for its apoptotic effect.

The apoptotic effect of AICAriboside is caspase-dependent and involves cytochrome c release from mitochondria

Jurkat cells were incubated with AICAriboside in the presence of the caspase inhibitor Z-VAD.fmk (200 μM) for 24 h and apoptosis was completely inhibited (Figure 3A). Furthermore, AICAriboside induced the appearance of the 85 kDa cleavage product of PARP, a hallmark of caspase activation, and a decrease of the precursor form of the executioner caspase-3 after 6 h of treatment (Figure 3B, lane 3). AICAriboside also induced the processing of caspase-9 after 3 h of treatment as shown by the appearance of a caspase-9 intermediate cleavage product (37 kDa; Figure 3B, lane 2). Bel-2 was also analysed by Western blot, showing no differences with AICAriboside treatment. To analyse the involvement of cytochrome c release in AICAriboside-induced apoptosis, cytosolic and mitochondrial fractions were obtained and the presence of cytochrome c was determined by Western blot. As shown in Figure 3(C), AICAriboside induced the appearance of cytochrome c in the cytosolic fraction of Jurkat cells 3 h after treatment, which was more marked at 6 h. As a control for protein loading, MAP kinase 2 was determined by Western blot. Cytochrome oxidase subunit II was also analysed as a control of mitochondrial contamination in the cytosolic fraction (Figure 3C).

AMPK is not involved in AICAriboside-induced apoptosis

Since it is well established that AICAriboside is an activator of AMPK [6], we wondered if apoptosis observed in Jurkat cells was due to AMPK activation. AMPK is activated by phosphorylation by the upstream kinase AMPK kinase at Thr-172, raising the activity of AMPK at least 50-fold [21]. This residue is also critical for the catalytic activity of AMPK [22]. Figure 4(A) shows that AMPK phosphorylation was increased when cells were treated with 10 μM oligomycin for 2 h in glucose-free medium. As a control for AMPK activation Jurkat cells were incubated with 10 μM oligomycin (Olig.) for 2 h in glucose-free medium, CT, untreated cells. Tubulin (55 kDa) was also analysed as a control for protein loading.
shows that AICArboside, ranging from 0.5 to 4 mM, did not induce AMPK phosphorylation, which was slightly decreased. Taken together, the above data show clearly that AMPK does not mediate the apoptosis induced by AICArboside in Jurkat cells.

**AICArboside induces ZMP accumulation, but ZMP is not necessary to induce apoptosis**

Since AICArboside is phosphorylated to ZMP, we analysed whether AICArboside treatment induces ZMP accumulation in Jurkat cells, as has been described for other cell types [2,6]. Nucleotide measurements were performed 3 h after treatment with AICArboside because cytochrome c was released (Figure 3C) and caspase-9 was already activated at this time (Figure 3B), without any significant decrease in cell viability (Figure 1B), which might interfere with the interpretation of the results. Under normal conditions ZMP was not detected by HPLC, but after incubation with 4 mM AICArboside a new peak appeared corresponding to ZMP with a concentration of 14.1 \( \pm 0.7 \) nmol/10^6 cells (Table 1). When cells were incubated with 1 or 4 mM AICArboside in the presence of adenosine, neither of which induces apoptosis, ZMP was not detected. When Jurkat cells were treated with 4 mM AICArboside plus 5-iodotubercidin, an inhibitor of adenosine kinase [25], which inhibits AICArboside phosphorylation [5,26], there was no accumulation of ZMP, as expected. However, AICArboside induced apoptosis even in the presence of 5-iodotubercidin (Table 1, bottom row). This clearly indicates that phosphorylation of AICArboside is not necessary to induce apoptosis, demonstrating that AMPK is not responsible for the apoptotic process. Other nucleotides remained practically unchanged under the conditions assayed except ATP and GTP, both of which increased significantly after incubation with 1 and 4 mM AICArboside. A significant increase in ATP, but not in GTP, was observed after treatment with 2 mM adenosine.

**DISCUSSION**

The present study shows that AICArboside induces apoptosis in Jurkat cells. Moreover, uptake of AICArboside through NBTr-sensitive transporters, but not its phosphorylation to ZMP, is necessary for this effect. We also show that activation of caspases is a prerequisite for AICArboside-induced apoptosis, since addition of Z-VAD.fmkn completely inhibits apoptosis, and caspase-9 is activated as early as 3 h, after addition of 4 mM AICArboside. Moreover, AICArboside induces cytochrome c release from mitochondria 3 h after treatment.

These results could seem surprising at first sight, since recent findings indicate that addition of AICArboside protects cells from apoptosis [11–14]. The results obtained by Stefanelli et al. [11] could be explained by their particular cell model (quiescent thymocytes), where ATP depletion inhibits glucocorticoid-induced apoptosis [27]. It has been reported that long-term incubation of cells with AICArboside significantly decreased ATP levels [5,15,28]. Therefore, the inhibition of apoptosis by AICArboside in this particular cell model could be explained by depletion of ATP levels. The anti-apoptotic effects of AICArboside in ceramide-induced apoptosis of astrocytes [13] and in hyperglycaemia-induced apoptosis of endothelial cells [14] are well explained by the blockade of fatty acid biosynthesis through AMPK activation. On the other hand, in agreement with our results, Pesi et al. [17] have shown that AICArboside causes apoptosis in two neuroblastoma cell lines. They also report that the erythrocytes of patients with Lesch–Nyhan syndrome, who accumulate Z-nucleotides [29], have higher IMP/GMP-specific 5'-nucleotidase activity than healthy controls [17], suggesting the involvement of AICArboside, and not ZMP or AICArboside triphosphate (‘ZTP’), in the pathogenesis of Lesch–Nyhan syndrome. However, these conclusions are very speculative and no definitive proof is presented. The toxic effect of AICArboside has also been reported in different cell types, including some lymphoid cell lines [1,15,16], but the mechanism responsible is not clear.

Release of cytochrome c from mitochondria is a central event in apoptosis [30]. Once released, cytochrome c binds to Apaf-1 and induces activation of caspase-9 [31]. Our results demonstrate that cytochrome c release from mitochondria is an early event in AICArboside-induced apoptosis. AICArboside induces activation of caspases 9 and 3. Thus the early AICArboside-induced cytochrome c release could be sufficient to trigger caspase activation.

AICArboside is a specific activator of AMPK in some cell lines and tissues [6–14]. However, AMPK is not activated by AICArboside in other cell lines [23]. We describe the apoptotic effect of AICArboside in Jurkat cells, but we rule out AMPK as a mediator of apoptosis for several reasons. First, addition of 4 mM AICArboside, a concentration that induces apoptosis in Jurkat cells, did not induce AMPK phosphorylation at Thr-172, an important step for AMPK activation [21]. In fact, we observed a slight decrease in the phosphorylation of AMPK after treatment with AICArboside. Nucleotide analysis showed that 4 mM AICArboside induced ZMP accumulation, and also an increase in ATP as early as 3 h after treatment. Since ATP inhibits AMPK [32], we hypothesize that AMPK kinase is also inhibited by ATP, which could explain why AMPK was slightly dephosphorylated in Jurkat cells. The decrease of AMPK phosphorylation was also observed with lower concentrations of AICArboside and does not appear to be important in terms of viability, since incubation with 1 mM AICArboside did not induce apoptosis in Jurkat cells. Second, phosphorylation of AICArboside to ZMP was not a prerequisite for induction of apoptosis in Jurkat cells. When this paper was in revision, Meisse et al. [35] showed that sustained activation of AMPK with AICArboside in FTO2B cells induces apoptosis. Since AMPK is not activated in Jurkat cells by AICArboside, we have described an AMPK-independent pathway for AICArboside-induced apoptosis. Meisse et al. [35] also induced apoptosis, overexpressing a constitutively active form of AMPK by adenoviral transfection of rat hepatocytes [35].

We found different ways to block the apoptosis induced by AICArboside. Z-VAD.fmkn, a specific inhibitor of caspases, and NBTr, an inhibitor of some nucleoside transporters, both completely blocked apoptosis in Jurkat cells. Seven nucleoside transporters have been described in mammalian cells: two equilibrative and five concentrative Na+-dependent [20]. Of these, only two, es (equilibrative) and N5 (concentrative), are inhibited by nanomolar concentrations of NBTr [20]. The high concentration of AICArboside required for induction of apoptosis could reflect the low uptake efficiency by Jurkat cells. We know that AICArboside induces apoptosis in B-cell chronic lymphocytic leukemia lymphocytes at a much lower concentration (0.2 mM) than in Jurkat cells [34]. Chronic lymphocytic leukaemia cells express equilibrative (ENT1 and ENT2) and concentrative (CNT2 and CNT3) nucleoside transporters whereas Jurkat cells lack the expression of CNT3 [35]. A difference in the transporters could contribute to explain the different sensitivity of cells to AICArboside. Furthermore, this difference can also be attributed to a higher metabolism of AICArboside in Jurkat cells. A more detailed study is necessary to characterize...
REFERENCES


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