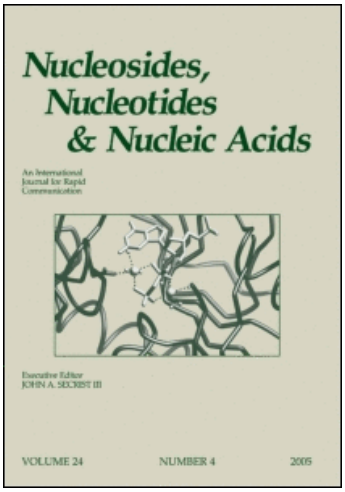

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M. Garcia-Gil[a]; F. Bertini[a]; R. Pesi[a]; V. Voccoli[b]; M. G. Tozzi[a]; M. Camici[a]
[a] Department of Biology, University of Pisa, Pisa, Italy [b] Institute of Neurosciences, CNR, Pisa, Italy

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5′-AMINO-4-IMIDAZOLECARBOXAMIDE RIBOSIDE INDUCES APOPTOSIS IN HUMAN NEUROBLASTOMA CELLS VIA THE MITOCHONDRIAL PATHWAY

M. Garcia-Gil, F. Bertini, and R. Pesi □ *Department of Biology, University of Pisa, Pisa, Italy*

V. Voccoli □ *Institute of Neurosciences, CNR, Pisa, Italy*

M. G. Tozzi and M. Camici □ *Department of Biology, University of Pisa, Pisa, Italy*

□ *5′-Amino-4-imidazolecarboxamide (AICA) riboside induces apoptosis in neuronal cell models. In order to exert its effect, AICA riboside must enter the cell and be phosphorylated to the ribotide. In the present work, we have further studied the mechanism of apoptosis induced by AICA riboside. The results demonstrate that AICA riboside activates AMP-dependent protein kinase (AMPK), induces release of cytochrome c from mitochondria and activation of caspase 9. The role of AMPK in determining cell fate is controversial. In fact, AICA riboside has been reported to be neuroprotective or to induce apoptosis depending on its concentration, cell type or apoptotic stimuli used. In order to clarify whether the activation of AMPK is related to apoptosis in our model, we have used another AMPK stimulator, metformin, and we have analysed its effects on cell viability, nuclear morphology and AMPK activity. Five mM metformin increased AMPK activity, inhibited viability, and increased the number of apoptotic nuclei. AICA riboside, which can be generated from the ribotide (an intermediate of the purine de novo synthesis) by the action of the ubiquitous cytosolic 5′-nucleotidase (cN-II), may accumulate in those individuals in which an inborn error of purine metabolism causes both a building up of intermediates and/or an increase of the rate of de novo synthesis, and/or an overexpression of cN-II. Therefore, our results suggest that the toxic effect of AICA riboside on some types of neurons may participate in the neurological manifestations of syndromes related to purine dismetabolisms.*

Keywords Neuroblastoma cells; Purine dismetabolisms; Cytochrome c; Metformin; AMP kinase

INTRODUCTION

In previous articles, we have demonstrated that 5′-amino-4-imidazolecarboxamide (AICA) riboside is toxic in neuronal cell lines and

Address correspondence to M. Camici, Department of Biology, University of Pisa, via S. Zeno 51, 56100, Pisa, Italy. E-mail: mcamici@biologia.unipi.it

that it acts on undifferentiated neuronal cells as a trigger of the apoptotic program.[1,2] In fact, the occurrence of apoptosis was demonstrated by the ladder pattern of DNA degradation observed in cells treated with the riboside,[1] and by the activation of effector caspase-3.[2] In order to exert its effect, AICA riboside must enter the cells and be phosphorylated to the ribotide,[2] which is known as a potent activator of the AMP-dependent protein kinase (AMPK).[3] Previous observations indicated that the treatment with AICA riboside induced an activation of AMPK on a human neuroblastoma cell line,[2] but whether this activation could play a role in the regulation of apoptosis remained to be ascertained. In this respect, in the last years, several articles have been published, in which the role of the activation of AMPK on the apoptotic pathway appears to be controversial. In fact, AMPK activation has been involved in both protection[4–7] and induction[8–11] of apoptosis. In this report, we describe the attempts made in order to characterize the apoptotic pathway activated by AICA riboside upstream of caspase-3, and to address the question of the possible involvement of the activation of AMPK in the induction of the apoptotic program.

MATERIALS AND METHODS

Materials. Fetal bovine serum was from Biochrom (Berlin, Germany); Dulbecco's modified Eagle's medium (DMEM), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), metformin and AICA riboside were purchased from Sigma (St. Louis, MO, USA); mouse monoclonal anti-cytochrome c antibody was from PharMingen (San Diego, CA, USA); rabbit anti-caspase 9 and rabbit anti-phosphoAMPK were from Cell Signalling Technologies Inc. (Beverly, MA, USA); antimouse IgG-HRP conjugate was from Dako (Glosturp, Denmark); antirabbit IgG-HRP conjugate was from Chemicon International (Temecula, CA, USA). SH-SY5Y human neuroblastoma cells were a kind gift of Dr. A. Arcangeli, University of Florence, Italy. All other reagents were of analytical grade.

Cell Culture and Treatments. SH-SY5Y neuroblastoma cells were grown as previously described.[2] The experiments were performed with 15000 cells plated in 96-well plates (viability) or 6×10^6 cells/dish (immunoblot); 24 hours after plating, cells were washed with serum-free DMEM and incubated in the presence of AICA riboside or metformin. Cell viability was assessed by the MTT assay as previously described.[2]

Nuclear Staining. Cells were fixed with paraformaldehyde and stained with Hoechst 33342 as in Giannecchini et al.,[13] and were examined by fluorescence microscopy.

Immunoblot Analyses. For AMPK, cells were scraped directly in lysis buffer and treated for the immunoblot analysis as described by Giannecchini et al.[14] For cytochrome c, cells have been scraped gently from plates, and

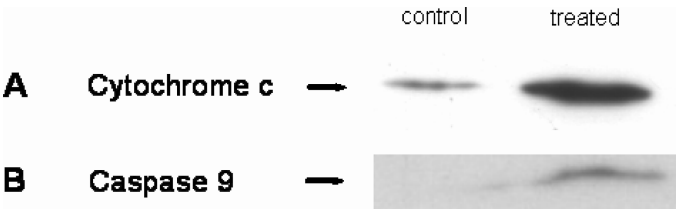

FIGURE 1 Effect of AICA riboside on cytochrome c release and caspase-9 activation. Immunoblots analyses of extracts of SH-SY5Y human neuroblastoma cells treated for 7 hours (cytochrome c) and 15 hours (caspase-9) with 1 mM AICA riboside.

treated as previously described.[13] For caspase 9, cells have been scraped from plates, washed with PBS, centrifuged at 300 g for 6 minutes and the pellet was resuspendend and treated with 50 mM Pipes/NaOH pH 6.5, 2 mM EDTA, 0.1% CHAPS, 5 mM DTT plus protein inhibitors as recommendad by Cell Signalling. Sixty μg of protein were separated in a 15% SDS-PAGE gel and transferred to PVDF membranes. Membranes were blocked for 2 hours with 5% non fat milk in PBS-0.1% Tween-20, incubated for 4 hours with anti-caspase 9 antibody and 2 hours in secondary antibody diluted 1:500 and 1:2000, respectively, in PBS-0.1% Tween-20, 2% non-fat milk. Immunoreactive protein was visualized by a chemiluminescence-detection kit. Each experiment was repeated at least three times.

RESULTS

AICA Riboside Induces Release of Cytochrome c from Mitochondria and Activation of Caspase-9 in SH-SY5Y Human Neuroblastoma Cells. Cellular extracts obtained after incubation with AICA riboside were tested in order to ascertain whether the treatment was able to induce translocation of cytochrome c from mitochondria to cytosol and activation of caspase-9. Immunoblot analysis with specific antibodies revealed the presence of cytochrome c in the cytosol of cells treated with AICA riboside for 7 hours, and that caspase-9 was proteolytically activated after 15 hours of AICA riboside treatment (Figure 1).

Metformin Induces Apoptosis and Activation of AMPK in SH-SY5Y Human Neuroblastoma Cells. Cells were cultured with either 1–5 mM metformin or 1 mM AICA riboside. Concentrations of metformin higher than 1 mM reduced cellular viability; the effect of 5 mM metformin started to be significant after 72 hours, when viability was 79.2 $\pm$ 5.3% compared to control (average $\pm$ SEM, n = 5). The effect of metformin was milder than that exerted by 1 mM AICA riboside; in fact, in cells cultured with the riboside, MTT reduction was 27.7 $\pm$ 2.6% of control cells. Metformin induced morphological changes typical of apoptosis since cells lost their neurites and became rounded with shrunken cytoplasm and condensed and

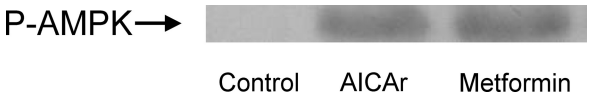

FIGURE 2 Effect of AICA riboside and metformin on the activation of AMPK. A typical immunoblot of extracts of SH-SY5Y human neuroblastoma cells treated for 3 hours with either 1 mM AICA riboside or 5 mM metformin is shown. The analysis was performed using an anti-phospho AMPK antibody.

fragmented nuclei showing intense fluorescence. Immunoblot analysis with anti-phosphoAMPK indicated that 3 hours treatment with AICA riboside and metformin brought about an activation of AMPK, evidenced by the increase in the state of phosphorylation of the protein (Figure 2).

DISCUSSION

Our results demonstrate that AICA riboside induces apoptosis in a human neuroblastoma cell line through the mitochondrial pathway. In fact, the appearance of apoptotic features is preceded by the release of cytochrome c from mitochondria to the cytoplasm, accompanied by the proteolytic activation of caspase-9, which is followed by the activation of effector caspase-3. Furthermore, a possible involvement of AMPK in the induction of apoptosis is suggested. Indeed, metformin, which has been reported to increase the rate of utilization of glucose through activation of AMPK,[15] is able to activate AMPK in our experimental model and to exert a cytotoxic and apoptotic effect, though to a lesser extent as compared to AICA riboside. The precise role of AMPK activity in cell fate is a controversial matter. Treatments that lead to sustained AMPK activation such as prolonged incubation with AICA riboside, metformin or incubation with constitutively active AMPK induce apoptosis in liver cells,[8] lymphocytic leukemia cells,[9] β-cells,[10] and neuroblastoma cells.[11] However, AICA riboside also can act independently of AMPK activation.[12] On the other hand, an activation of AMPK has been reported to protect against apoptosis induced by glucose deprivation in hippocampal neurons,[4] by dexamethasone in thymocytes,[5] by fatty acids in astrocytes,[6] or by hyperglycemia in human umbelical-vein endothelial cells.[7] Therefore, though the details of the mechanism of action of AMPK activation in induction of apoptosis in neuroblastoma cells require further research, it is conceivable that the effect of AMPK activation on cell survival may depend upon the cell type, the environmental conditions and on the duration of kinase activation.

Finally, some speculations can be made on the possible pathophysiological significance of the observed AICA riboside-induced apoptosis in human neuroblastoma cells. Accumulation of AICA riboside mono- and tri-phosphate is associated with hypoxanthine-guanine phosphoribosyltransferase deficiency and other disorders of purine metabolism and has been attributed to the increased rate of the "de novo" purine synthesis in these

conditions,[16] which is accompanied in the Lesch-Nyhan syndrome, by a hyperactivity of cytosolic 5′-nucleotidase (cN-II), the enzyme responsible for the conversion of AICA ribotide (an intermediate of de novo synthesis) to the riboside.[1] Recently, a massive excretion of AICA riboside and accumulation of its mono-, di-, and triphosphate forms has been found in red blood cells of a patient with a deficiency in the activity of AICAR transformylase/IMP cyclohydrolase, due to a mutation in one allele and a frameshift in the other. The deficit in the activity of the enzyme was accompanied by severe neurological defects, and congenital blindness.[17] Therefore, our results suggest that the toxic effect of AICA riboside on some types of neurons may participate in the neurological manifestations of syndromes related to purine dismetabolisms.

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