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AICAR Antiproliferative Properties Involve the AMPK-Independent Activation of the Tumor Suppressors LATS 1 and 2

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Abstract

AICAR (Acadesine) is a pharmacological precursor of purine nucleotide biosynthesis with anti-tumoral properties. Although recognized as an AMP mimetic activator of the protein kinase AMPK, the AICAR monophosphate derivative ZMP was also shown to mediate AMPK-independent effects. In order to unveil these AMPK-independent functions, we performed a transcriptomic analysis in AMPKα1/α2 double knockout murine embryonic cells. Kinetic analysis of the cellular response to AICAR revealed the up-regulation of the large tumor suppressor kinases (Lats) 1 and 2 transcripts, followed by the repression of numerous genes downstream of the transcriptional regulators Yap1 and Taz. This transcriptional signature, together with the observation of increased levels in phosphorylation of Lats1 and Yap1 proteins, suggested that the Hippo signaling pathway was activated by AICAR. This effect was observed in both fibroblasts and epithelial cells. Knockdown of Lats1/2 prevented the cytoplasmic delocalization of Yap1/Taz proteins in response to AICAR and conferred a higher resistance to the drug. These results indicate that activation of the most downstream steps of the Hippo cascade participates to the antiproliferative effects of AICAR.

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Introduction

AICAR (5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranoside) is a pharmacologically active compound entering cells *via* purine transporters and being converted to its active-monophosphorylated form ZMP by adenosine kinase [1,2]. ZMP mimics AMP as a low-energy charge signal by activating the heterotrimeric AMP-activated protein kinase (AMPK), which promotes integrated regulatory functions at the cellular and tissular levels [3].

In mammal cells, an AICAR-induced cytotoxic response is observed, leading to both apoptotic and non-apoptotic death mechanisms [4–8]. Interestingly, AICAR is more cytotoxic for aneuploid cells compared to their euploid counterparts [6,9], which is of a great therapeutic interest since aneuploidy is observed in human in 90% of solid tumors and 85% of hematopoietic malignancies [10]. Moreover, AICAR showed antitumoral properties *in vivo* in different animal models [6,11,12] and is well tolerated in mice and human, even at elevated and repeated administration doses [13,14]. AICAR has been subjected to Phase I/II clinical trials for treating chronic lymphocytic leukemia [14].

Although AMPK activation has been initially recognized as a prevalent effect of AICAR, recent data indicated that many effects mediated by this compound are in fact AMPK-independent. These include inhibition of proliferation and limited tumor growth *in vivo*

Abbreviations: AICAR, 5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranoside; AMPK, adenosine monophosphate-activated protein kinase; CTGF, connective tissue growth factor (CCN2); CYR61, cysteine-rich angiogenic protein 61; HPRT, hypoxanthine-guanine phosphoribosyl transferase; LATS, large tumor suppressor kinase; MEFs, murine embryonic fibroblasts; YAP1, Yes-associated protein 1; TAZ, transcriptional coactivator with PDZ-binding motif; ZMP, AICAR monophosphate

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[2,5,15–17]. These intriguing properties of AICAR therefore motivate the search for alternative targets of its metabolic derivative ZMP that could be exploited therapeutically.

In order to get further insight on the AMPK-independent action of AICAR, we carried out a kinetic analysis of the effects of AICAR on the transcriptome changes in murine embryonic fibroblasts (MEFs) invalidated for AMPK (α1 and α2 subunits) [18]. We identified *Lats1* and *Lats2* as early AICAR-induced genes and showed that the encoded proteins (herein referred as to Lats1/2) are potent mediators of the cell response to this drug. Lats1/2 proteins are downstream transducers of the core Hippo pathway known to play a pivotal role in cells and tissues [19,20]. Lats1/2 display pleiotropic functions, which include the inhibition of the two related co-transcriptional factors Yap1 (Yes-associated protein 1) and Taz (Transcriptional coactivator with PDZ-binding motif). Knockdown of Lats1/2 established that some of the cellular effects triggered by AICAR result of the activation of this central pathway controlling cell cycle and density. These findings provide a rationale to analyze the effects of AICAR and motivate the research of derivatized compounds of AICAR with therapeutic perspectives.

Materials and Methods

Reagents

Culture media was from Invitrogen. Specific antibodies were as follows: Taz (#560235) (BD Pharmingen); actin (#A2668) (Sigma); AMPKα (#2532), PARP (#9542), LATS2 (#13646), LATS1 (#3477), phospho-(Thr1079)-LATS1 (#8654), LC3A/B (#4108), cleaved-(Asp175)-caspase-3 (#9604 and 9664), YAP1/TAZ (#8418), YAP1 (#14074 and #4912), phospho-(Ser127)-YAP1 (phospho-(-Ser112)-Yap1 in mice) (#4911) were from Cell Signaling; LATS2 (#NB200–199, NovusBio); DAPI and secondary antibodies labeled with AlexaFluor488 or with AlexaFluor555 were from ThermoFisher Scientific. Primers (Table S1) and siRNA (see below) were from Sigma. Control siRNA was from Cell Signaling (#6568). AICAR (#A611700, Toronto Research Chemicals, Canada) was solubilized in water and filtered at 0.22 µm. Its concentration was determined spectrophotometrically (A_{269}; pH = 7; ε_M = 12,700 M^{-1}/cm^{-1}).

Cell Culture

Human RPE-1 (ATCC; CRL-4000) cells were grown in DMEM, 4.5 g/L glucose supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. Wild-type and knock-out mouse embryonic fibroblasts (MEFs) for both AMPKα1 and AMPKα2 subunits (MEF-dKO) were obtained from the U1016-INSERM, Paris (Laderoute *et al.* [18]) and the absence of AMPKα in MEF-dKO was confirmed by Western blot (see text). The WST-1 cell viability assay was performed in 96-well plates as previously described [2]. Cell proliferation assays were performed in 24-well plates and cells were counted using a Multisizer 4 Coulter Counter (Beckman). Each time point was done in triplicate, and the averages ± STD were calculated. Experiments were repeated three times.

Preparation of cRNA, Microarray Hybridization and Gene Expression Profiling

Microarray analysis was performed at the Affymetrix transcriptome Platform, CHRU-Inserm U1040, Montpellier France (http://www.chu-montpellier.fr/fr/irmb/). MEF-dKO were grown in complete DMEM medium in the presence or absence of 1 mM AICAR and harvested at the indicated times (Final density ~15,000 cells/cm^2). Total RNA was extracted using the RNeasy kit (Qiagen, Santa Clarina, CA) and the quality of each sample was assessed on a Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA). Affymetrix Gene Chip 3`IVT Express Kit Bundle was used to generate amplified RNA. cRNAs were hybridized to Mouse MG-430 PM Array Strip Kit (44,000 probesets). All steps were performed according to the standard protocol of the manufacturer. Fluorescence intensities were quantified and analyzed using the Expression Console v 1.2 software. The converted digital intensity values were then converted into cell intensity files using the Affymetrix GeneAtlas Software. The fold change in gene expression corresponds to the ratio between each incubation times with AICAR and control without AICAR. Significance analysis of microarrays (SAM) analysis was applied in the different samples with 400 permutations, a fold change of 2 and a false discovery rate (FDR) < 3.55% (see Table S2A). All steps were conducted according to the MIAME (Minimum Information About a Microarray Experiment) checklist [21]. Annotation of genes was performed using NetAffx (http://www.affymetrix.com). Affymetrix IDs were submitted to the DAVID analysis tool (http://david.abcc.ncifcrf.gov/home.jsp), the Netaffx Analysis Center (https://www.affymetrix.com/analysis/compare/index.affx) and the KEGG pathway database (www.genome.jp/kegg/pathway.html) to depict biological processes associated with the functions of regulated genes. Data files were deposited in the Gene Expression Omnibus (GEO) database (accession number, GSE106460; www.ncbi.nlm.nih.gov/geo).

Small Interfering RNA Knockdown

Cells were plated at a density of 5.10^4 cells per well in six-well plates. Small interfering RNA (siRNA) against human and mouse LATS1 (sihmLATS1): CACGGCAAGAUAGCAUGGAUU; human and mouse LATS2 (sihmLATS2-y): GAAGAUUGUAUUUAUG GUAAA; mouse AMPKα1/2_human AMPKα2 (simAMPKα1/2 sihAMPKα2): GAGAAGCAGAAGCACGACG; human AMPKα1 (sihAMPKα1): AUGAUGAAAUUACAGAAGCCA were from Sigma. Non-targeting siRNA (#D-001810-01-20) was from Dharmacon (Lafayette, CO). Transfection was performed for 6–15 h using lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocol, with total amounts of siRNA at a final concentration of 10–60 nM, as indicated in the figure legends.

Immunoblot

Cells in culture dishes were collected using a rubber policeman and snap frozen. Proteins were extracted at 4°C with RIPA buffer containing phosphatase inhibitors (PhosStop EasyPack, ROCHE) and protease inhibitors (Complete ULTRA Tablets, mini EasyPack, ROCHE). Protein content was determined by using a BCA protein assay kit (Pierce). Proteins were resolved on 7 or 10% SDS-PAGE, transferred to a PVDF blotting membrane (Hybond P 0.45 µm PVDF membrane; Amersham) and probed using primary antibodies. Secondary antibodies coupled to HRP were used for revelation. Densitometric analyses were carried out using ImageJ (http://rsbweb.nih.gov/ij/download.html) and results were normalized to the actin loading control.

Real-Time PCR

Cells in culture dishes were solubilized in Trizol (Life Technologies) and frozen at −80°C until use. RNA extraction, quantification and gene expression analyses by RT-qPCR were performed as