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AICAR suppresses IL-2 expression through inhibition of GSK-3 phosphorylation and NF-AT activation in Jurkat T cells

Bong Sook Jhun [a,b], Young Taek Oh [a,b], Jung Yeon Lee [a,b], Yoon Kong [c], Kyung-Sik Yoon [a,b], Sung Soo Kim [a,b], Hyung Hwan Baik [a], Joohun Ha [a,b], Insug Kang [a,b,*]

[a] *Department of Biochemistry and Molecular Biology, School of Medicine, Kyung Hee University, Seoul 130-701, Republic of Korea*
[b] *Medical Science and Engineering Research Center for Bioreaction to Reactive Oxygen Species, School of Medicine, Kyung Hee University, Seoul 130-701, Republic of Korea*
[c] *Department of Molecular and Cellular Biology, School of Medicine, Sungkyunkwan University, Suwon 440-746, Republic of Korea*

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Abstract

We examined the effect of 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), the dephosphorylated form of AICA ribotide (also termed "ZMP"), an intermediate of purine biosynthesis, on interleukin (IL)-2 production in T cells. AICAR inhibited IL-2 production in Jurkat T cells and peripheral blood lymphocytes activated with PMA plus ionomycin (PMA/Io) or with monoclonal anti-CD3 plus anti-CD28. Pretreatment with 5′-iodotubercidin, an adenosine kinase inhibitor, enhanced AICAR suppression of IL-2 production, suggesting that AICAR, not ZMP, is responsible for IL-2 suppression. We then showed that AICAR inhibited PMA/Io-induced IL-2 mRNA expression and IL-2 promoter activation. AICAR inhibited DNA binding and transcriptional activation of NF-AT and to a lesser extent AP-1, but not NF-κB, in PMA/Io-activated Jurkat cells. Finally, we found that AICAR inhibited PMA/Io-induced phosphorylation of GSK-3 but not phosphorylation of ERK1/2, p38, and JNK. These results suggest that AICAR exerts its immunosuppressive effect in activated Jurkat cells by inhibiting GSK-3 phosphorylation and NF-AT activation.

Keywords: AICAR; Jurkat T cells; PMA; Ionomycin; IL-2; NF-AT; GSK-3; Immunosuppression

T-cell activation through the T-cell receptor (TCR)/CD3 complex and the CD28 costimulatory molecule results in enhanced induction of the autocrine growth factor interleukin 2 (IL-2), governing expansion of antigenic T cells [1–3]. Inducible expression of IL-2 is tightly regulated by multiple transcription factors that bind at distinct sites on the IL-2 promoter [4], including activator protein-1 (AP-1), nuclear factor-κB (NF-κB), and the nuclear factor of activated T cells (NF-AT) [4,5]. Binding sites for NF-AT have also been found within the promoter regions of other cytokine genes including IL-4, IL-5, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α [6]. The activity of NF-AT is regulated by its phosphorylation state. In resting T cells, NF-AT is phosphorylated and retained in the cytoplasm. Upon T-cell stimulation, calcineurin, a Ca^{2+}/calmodulin-dependent Ser/Thr phosphatase, dephosphorylates NF-AT, which then can enter the nucleus and bind DNA, activating IL-2 transcription [6,7]. Although less is known about NF-AT inactivation, glycogen synthase kinase-3 (GSK-3) exerts regulatory actions opposed to those of calcineurin [8]. Active GSK-3 directly phosphorylates NF-AT, facilitating NF-AT nuclear export and termination of IL-2 production [6–8]. Since T lymphocyte proliferation is primarily mediated by IL-2, inhibition of IL-2 production is a central

* Corresponding author. Fax: +82 2 965 6349.
E-mail address: iskang@khu.ac.kr (I. Kang).

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mechanism of action of several immunosuppressants, including cyclosporine A [1,9].

5-Aminoimidazole-4-carboxamide ribonucleoside (AICAR) is found in the blood or urine of patients suffering an inborn error of purine metabolism [10,11]. AICAR is internalized and becomes phosphorylated by adenosine kinase to form AICAR monophosphate (AICA ribotide, ZMP), an intermediate in the late steps of de novo purine biosynthesis [12]. Thus, AICAR has been used to restore purine nucleotide pools in ischemic myocardium [13] and as a specific activator of AMP-activated protein kinase (AMPK), because ZMP mimics the effects of AMP on AMPK [14]. AMPK is an important regulator of energy homeostasis and effects of AICAR on lipid and glucose metabolism have been widely studied [15]. Besides, AICAR exerts various other effects in different cell types: it regulates cell proliferation, apoptosis, and inflammation via AMPK-dependent and -independent mechanisms [16–20].

Although AICAR has been reported to exert both anti-apoptotic and pro-apoptotic actions in human T lymphocytes [17,18,21], its effect on cytokine production in activated T cells is not known. Thus, we examined the effect of AICAR on IL-2 production and the molecular mechanisms of its action in activated human T cells. We found that AICAR inhibited IL-2 production in activated human leukemic Jurkat cells and peripheral blood lymphocytes (PBLs), and that AICAR inhibited GSK-3 phosphorylation and NF-AT activation in PMA/Io-stimulated Jurkat cells.

Materials and methods

Materials. RPMI 1640 medium and other cell culture products were obtained from Life Technologies (Grand Island, NY). AICAR was obtained from Toronto Research Chemicals (Toronto, Ontario, Canada). 5′-Iodotubercidin was purchased from Biomol Research Labs (Plymouth Meeting, PA). PMA, ionomycin, NBTI, MRS1523, and all other chemicals used were purchased from Sigma (St. Louis, MO). An IL-2 ELISA kit, and anti-CD3 (UCHT1) and anti-CD28 monoclonal antibodies (mAbs) were obtained from R&D Systems (Minneapolis, MN). IFN-γ and TNF-α ELISA kits were from Biosource (Camarillo, CA). Antibodies against phospho-ERK1/2, phospho-p38, ERK1/2, phospho-JNK, and phospho-GSK-3β were purchased from Cell Signaling Technology (Beverly, MA); antibodies against p38, JNK1, and GSK-3β came from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture and treatment. Jurkat T cells (American Type Culture Collection) were cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum in a humidified 5% (v/v) CO_2 atmosphere at 37 °C. Human peripheral blood mononuclear cells (PBMCs) from healthy adult donors were isolated by density-gradient centrifugation using Ficoll–Paque PLUS (Amersham Biosciences) as described [22]. Isolated cells were plated in culture flasks at a density of 2×10^6/mL and incubated at 37 °C in a 5% CO_2 atmosphere for 2 h to remove adherent macrophages. The resulting human PBLs were resuspended in complete RPMI medium and used within 24 h. For experiments, PBLs in complete medium or Jurkat cells in serum-free medium were stimulated with either PMA (100 nM) plus ionomycin (1 μM) (PMA/Io) or immobilized anti-CD3 (2 μg/mL) plus anti-CD28 (2 μg/mL) mAb (CD3/CD28) in the presence or absence of AICAR or other drugs. Primary T cells (mixtures of $CD4^+$ and $CD8^+$ cells) were isolated from human PBMCs using autoMACS (autoMACS; Miltenyi Biotec, Germany) as described [23], and tested for the cytotoxicity of AICAR.

Enzyme-linked immunosorbent assay. For analyses of cytokines, Jurkat cells or PBLs (2×10^6 cells/mL) were treated with AICAR for 1 h followed by PMA/Io or CD3/CD28 for 24 h. Cytokine levels in culture supernatants were measured with IL-2, IFN-γ, and TNF-α ELISA kits according to the manufacturer's instructions. The concentrations of cytokines in each sample were calculated from a standard curve prepared using known concentrations of recombinant cytokines.

Luciferase assay. Jurkat cells (2×10^6 cells/well) were transfected with 2 μg of luciferase reporter plasmids (IL-2-luc, NF-AT-luc, and AP-1-luc were gifts from Dr. G.R. Crabtree, Stanford University, CA; NF-κB-luc was a gift from Dr. D. Baltimore, California Institute of Technology, CA) and 0.2 μg of the pSV-β-galactosidase plasmid (Promega) using GenePORTER transfection reagent (Gene Therapy Systems) according to the manufacturer's protocol. After 24 h of transfection, cells were deprived of serum for 4 h, preincubated with 1 mM AICAR for 1 h, and then stimulated with PMA/Io for 16 h. After stimulation, the cells were washed, lysed, and analyzed using a luciferase assay system (Promega) with a VICTOR luminometer (Perkin-Elmer). Luciferase activity was normalized to β-galactosidase activity as described [24].

Preparation of nuclear extracts and electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared as described previously [25]. In brief, cells (5×10^6) were washed with ice-cold PBS and then were gently scraped in 0.2 mL ice-cold lysis buffer. The nuclei were then pelleted and resuspended in 50 μL ice-cold nuclear extraction buffer. Two double-stranded oligonucleotide probes containing a consensus binding sequence for either NF-AT (5′-GGAGGAAAAACTGTTTCATA-3′), AP-1 (5′-CGCTTGATGACTCAGCCGGAA-3′), or NF-κB (5′-AGTTGAGGGGACTTTCCCAGGC-3′) were labeled with [α-^{32}P]CTP using a Rediprime II labeling system (Amersham Biosciences). For mobility shifts, the nuclear extracts (10 μg) were incubated with ^{32}P-labeled oligonucleotide (~50,000 cpm) in 20 μL of binding buffer containing 2 μg poly(dI–dC) as nonspecific competitor. Reactions proceeded for 30 min at room temperature and the resulting complexes were then separated on 4% polyacrylamide gels in Tris–glycine/EDTA buffer at pH 8.5. The gels were dried and visualized by autoradiography.

RT-PCR. Total RNA was extracted from cultured cells using TRI Reagent (Sigma); RT-PCR was performed using an RT-PCR kit (Bioneer, Korea) according to the manufacturer's instructions. cDNA was synthesized by incubating total RNA samples at 57 °C for 10 min, followed by 37 °C for 60 min and 94 °C for 5 min. For IL-2 (467 bp), the forward primer used was 5′-ATGTACAGGATGCAACTCCTG TCTT-3′; the reverse primer used was 5′-GTTAGTGTTGAGAT GATGCTTTGAC-3′. For GAPDH (598 bp), which was used as a reaction standard, the forward primer used was 5′-CCACCCATGGC AAATTCCATGGCA-3′, and the reverse primer used was 5′-TCTAG ACGGCAGGTCAGGTCCACC-3′. Thirty cycles of PCR amplification were performed at 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min, then stopped by final extension for 10 min at 72 °C. PCR products were visualized on a 2% agarose gel by ethidium bromide staining.

Immunoblotting. Protein extracts (50 μg) were separated by 10% SDS–polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. After transfer, membranes were incubated for 1 h with primary antibodies, followed by incubation with a secondary antibody. The blots were detected with an ECL kit (Amersham Biosciences).

Statistical analyses. All data are expressed as means ± SD of more than three independent experiments conducted in triplicate. Statistical analysis was performed using Student's t test and an analysis of variance (one-way ANOVA). The accepted level of significance was $P < 0.05$.