5-Aminooimidazole-4-carboxamide-1-β-D-ribofuranoside Inhibits Cancer Cell Proliferation in Vitro and in Vivo via AMP-activated Protein Kinase*

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5-Aminooimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) is widely used as an AMP-kinase activator, which regulates energy homeostasis and response to metabolic stress. Here, we investigated the effect of AICAR, an AMPK activator, on proliferation of various cancer cells and observed that proliferation of all the examined cell lines was significantly inhibited by AICAR treatment due to arrest in S-phase accompanied with increased expression of p21, p27, and p53 proteins and inhibition of PI3K-Akt pathway. Inhibition of in vivo growth of cancer cells was mirrored in vivo with increased expression of p21, p27, and p53 and attenuation of Akt phosphorylation. Anti-proliferative effect of AICAR is mediated through activated AMP-activated protein kinase (AMPK) as lactotubericidin and dominant-negative AMPK expression vector reversed the AICAR-mediated growth arrest. Moreover, constitutive active AMPK arrested the cells in S-phase by inducing the expression of p21, p27, and p53 proteins and inhibiting Akt phosphorylation, suggesting the involvement of AMPK. AICAR inhibited proliferation in both LKB and LKB knock-out mouse embryo fibroblasts to similar extent and arrested cells at S-phase when transfected with dominant negative expression vector of LKB. Altogether, these results indicate that AICAR can be utilized as a therapeutic drug to inhibit cancer, and AMPK can be a potential target for treatment of various cancers independent of the functional tumor suppressor gene, LKB.

AMP-activated protein kinase (AMPK) is a highly conserved serine/threonine protein kinase. It is a heterotrimer containing a catalytic (α) and two regulatory subunits (β and γ), each of which have at least two isoforms (1). AMPK is called the “fuel gauge” of the biological system, because it is activated under conditions that deplete cellular ATP and elevate AMP levels, such as glucose deprivation, heat shock, hypoxia, and ischemia (2, 3), and also by hormones like leptin (4), adiponectin (5), and interleukin-6 (7). Upon activation, AMPK phosphorylates and inactivates a number of metabolic enzymes involved in ATP-consuming pathways like fatty acid, cholesterol synthesis, and protein synthesis that include enzymes like acetyl-Coenzyme A carboxylase (ACC), fatty acid synthase, 3-hydroxy-3-methylglutaryl-CoA reductase, and mammalian target of rapamycin (mTOR) and activates ATP-generating process like fatty acid oxidation and glucose uptake (8). The mechanisms of activating AMPK include direct allosteric binding of AMP to the β subunits and phosphorylation, catalyzed by an upstream AMP kinase (AMPKK), recently identified to be LKB1 (STK11) (9–11). Recent studies have demonstrated that AMPK can also be activated by other stimuli that do not cause a detectable change in the AMP/ATP ratio, like hyperosmotic stress and pharmacological agents like thiazolidinediones, metformin, and 5-aminooimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) (11–14).

Activation of AMPK has been related with protection from injury and apoptosis caused by myocardial ischemia (15, 16) and apoptosis due to metabolic stress (17–19). In these scenarios, AMPK has been proposed as an anti-apoptotic molecule. However, recent reports have indicated anti-proliferative and pro-apoptotic action of activated AMPK using pharmacological agents or AMPK overexpression. AMPK activation has been shown to induce apoptosis in human gastric cancer cells (20), lung cancer cells (21), prostate cancer (22), pancreatic cells (23), and hepatic carcinoma cells (24) and enhance oxidative stress induced apoptosis in mouse neuroblastoma cells (25), by various mechanisms that includes inhibition of fatty acid synthase pathway and induction of stress kinases and caspase 3.

AMPK is an anti-growth molecule because of its relationship with two tumor suppressor genes: LKB and TSC2 (tuberous sclerosis complex 2). LKB functions as an upstream kinase (AMPKK) that activates AMPK (26). LKB mutations result in Peutz-Jeghers syndrome, which results in predisposition to cancers of the colon, pancreas, breast, and other sites (27–29). Mutations of LKB1 typically occur in the catalytic domain, leading to loss of its kinase activity and presumably a failure to phosphorylate and activate AMPK (30). TSC2 forms a complex with TSC1 and inhibits mTOR, leading to inhibition in protein synthesis and negative regulation of cell size and growth (31). Mutations of TSC1-TSC2 causes tuberous sclerosis, which is associated with hamartomatous polyps in multiple tissues and an increased risk of cancers (32).

In the present study we have investigated the effect of AICAR on cell proliferation in vivo and in vitro in various cancer cell lines. AICAR is converted to its triphosphorylated form ZMP, inside the cell, by an adenosine kinase (14), which acts as an AMP analogue and activates AMPK and its upstream kinase LKB without affecting the ATP:AMP ratio in the cell (14). AICAR-mediated AMPK activation was found to be a proficient inhibitor of cell proliferation and the mechanism of its anti-proliferative effect may be mediated via inhibition of PI3K-Akt...
pathway and increased expression of cell cycle inhibitory proteins p21, p27, and p53, thereby exhibiting potential as an anti-cancer drug.

**MATERIALS AND METHODS**

*Reagents and Cell Culture*—DMEM/F-12, DMEM/4.5 g of glucose medium, fetal bovine serum (FBS), and Hanks’ balanced salt solution were obtained from Invitrogen as was RPMI 1640. AICAR was purchased from Toronto Research Chemicals (Ontario, Canada). Iodotubericidin was obtained from Calbiochem. [3H]Thymidine ribotide ([3H]TdR) was purchased from PerkinElmer Life Sciences. Propium iodide, 3-[(4,5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide (MTT), and transfection reagent, FuGENE, were purchased from Roche Applied Science. The enhanced chemiluminescence (ECL) detecting reagent was from Amersham Biosciences, and the luciferase assay system was from Promega (Madison, WI). C6 glioma, T98G astrocytoma, U87MG astrocytoma, MCF-7 breast cancer, and PC-3 prostate carcinoma cell lines were obtained from ATCC (Rockville, MD), hematological cancer cell lines (CEM T-lymphoblast cells, K-562 chronic myelogenous leukemia cells) were a kind gift from Dr. Tomi P. Makela (Institute of Biomedicine and Helsinki University Central Hospital, Biomedicum Helsinki, University of Helsinki, Finland) and were maintained in DMEM/4.5 g of glucose with 10% FBS, essential amino acids, and antibiotics. All treatments were done in the presence of serum.

*Cell Culture*—C6 glioma cells, T98G, U87MG, and PC-3 were maintained in DMEM/F-12 medium supplemented with 10% FBS and antibiotics. MCF-7 cells were maintained in DMEM/4.5 g of glucose with 10% FBS. CEM and K-562 were maintained in RPMI 1640 supplemented with 10% FBS. LKB knock-out and wild-type mouse embryo fibroblasts (MEFs) were a kind gift from Dr. Tomi P. Makela (Institute of Biomedicine and Helsinki University Central Hospital, Biomedicum Helsinki, University of Helsinki, Finland) and were maintained in DMEM/4.5 g of glucose with 10% FBS, essential amino acids, and antibiotics. All treatments were done in the presence of serum.

*Thymidine Incorporation*—Proliferation of cells was determined by [3H]thymidine ribotide ([3H]TdR) incorporation into DNA. 1 × 10^6 cells per well of adherent cell lines (C6, MCF-7, and PC3) and 0.25 × 10^6 cells/well of suspension cell lines (NALM-6, CEM, CEMP, and K562) were plated in respective medias. Cells were incubated for 18–24 h with...
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or without the presence of AICAR at the indicated concentrations. Each group was exposed to 37 kBq/ml [methyl-3H]thymidine in the same medium for 6 h. The adherent cells were fixed by 5% trichloroacetic acid and lysed in SDS/NaOH lysis buffer overnight. Radioactivity was measured by Beckman LS8001 liquid scintillation counter (Canada). Suspension cell culture was harvested by cell harvester (Packard instrument Co., Meriden, CT), and radioactivity was measured by 1450 microbeta liquid scintillation counter (PerkinElmer Life Sciences).

Clonogenic Assay—Cells were treated with AICAR for 18–24 h, trypsinized, counted, and 300 cells/100-mm plate were plated. The cells were allowed to form colonies, and media was changed every third day for 2–3 weeks. The colonies were stained with MTT and enumerated (33).

Flow Cytometry Assessment of Cell Cycle—Cellular DNA content was assessed by flow cytometry. Cells were cultured in 6-well plates and treated with AICAR or transfections were performed. Cells attached to the plate were collected with trypsin, washed, and resuspended in 100 μl of PBS, and 5 ml of 70% ethanol was added slowly while continuous vortexing of cells and were fixed overnight. Next day, cells were spun, washed, and suspended in 400 μl of PBS with addition of 10 mg/liter RNase A and 75 μM propidium iodide. Cells were acquired by flow cytometry (BD Biosciences FACS Calibur flow cytometer) using ModFit LT software.

Immunoblot—After a stipulated time of incubation in the presence or absence of AICAR, cells were scraped, washed with Hanks’ buffer, and sonicated in 50 mM Tris–HCl (pH 7.4) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 5 μg/ml antipain, 5 μg/ml pepstatin A, and 5 μg/ml leupeptin). Proteins (50 μg/lane) were resolved by SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked for 1 h at 5% nonfat dry milk in TBBS (20 mM Tris, 500 mM NaCl, and 0.1% Tween 20, pH 7.5) and incubated overnight in primary antibody (p21, p27, p53, Akt, β-actin, mTOR, PCNA at 1:2000 dilution) containing 5% nonfat dry milk for non-phospho antibodies and containing 5% albumin for phospho-antibodies (Akt-p, mTOR-p at 1:1000 dilution). The blots were washed four times with TBBS (5 min/wash) and incubated for 45 min at room temperature with respective horseradish peroxidase-conjugated secondary antibody (1:5000). The blots were washed three times in TBBS and once in 0.1M PBS (pH 7.4) at room temperature; protein expression was detected with ECL.

Animals—Adult male Wistar rats weighing 200–250 g were purchased from Charles-River Laboratories. Animals were maintained, and all protocols were approved by the animal use committees of the Medical University of South Carolina in compliance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health).

Tumor Implantation—C6 glioma cells were prepared fresh from culture to ensure optimal viability of cells during tumor inoculation. The cells were trypsinized, and 10^6 tumor cells prepared in 100 μl of PBS were injected subcutaneously in the lateral side of the right hind leg of the rats, after shave and sterile preparation. On the 5th day of implantation 100 mg/kg body weight/day of AICAR was given intraperitoneal until the 14th day, when the animals were sacrificed and the tumor was excised, weighed, and fixed in formalin (34).

Immunohistochemistry—Tumor sections were processed as previously described (35). In brief, deparaffinized and rehydrated sections were microwaved for 10 min in antigen unmasking fluid (Vector Laboratories, Burlingame, CA), treated with 3% hydrogen peroxide in methanol to eliminate endogenous peroxidase activity and blocked to reduce nonspecific staining. Sections were incubated overnight with primary antibody (1:100) followed by tyramide signal enhancement technique (Renaissance TSA for Immunocytochemistry, PerkinElmer Life Sciences) per manufacturer’s instructions. After washing, slides were air-dried and mounted with aqueous mounting media (Vectorshied, Vector Laboratories). The sections were examined under a fluorescence microscope (Olympus BX-60) with an Olympus digital camera (Optronics, Goleta, CA) using a dual band pass filter. Images were captured and processed using Adobe Photoshop 7.0.

Transfection Studies—Plasmids were purified using the endotoxin-free plasmid midi prep kit (Qiagen). For transient transfections, C6 glioma cells were seeded in 6-well plates and grown to 60–80% confluence in DMEM/F-12 plus 5% FBS without antibiotics and transfected using FuGENE reagent. 1–3 μg of AMPK DN or AMPKa1CA or AMPKa2 CA expression vector along with 1 μg of eGFP expression vector or insertless expression vector (pcDNA3.1) were used for transfecting. Cells were treated with AICAR for 24 h and processed for GFP-gated DNA analysis by flow cytometry. Similarly, LKB1 wild type (1 μg) and dominant negative (1 μg) along with STRADα (0.5 μg) and MO25α (0.5 μg) expression vectors were used for transfection studies.

Antisense Experiments—To decrease the levels of endogenous AMPK, C6 glioma cells were transfected for 48 h with 25 μM phosphothiorated antisense (AS) oligonucleotide (5'-CGCGCCGTCGTCCG-GTCTCTGCG-3') directly against both the α1- and α2-subunits of AMPK (36, 37) and a missense (MS) oligonucleotide (5'-CTCCCCG-GTCTCGGCGG-3') along with eGFP expression vector (36). Oligonucleotides were transfected with FuGENE reagent per the manufacturer’s instructions. The cells were then treated with AICAR for 24 h and analyzed for cell cycle analysis by flow cytometry.

P38K Activity—After 2 h of AICAR (1 mM) treatment, cells were lysed with ice-cold lysis buffer containing 1% v/v Nonidet P-40, 100 mM NaCl, 20 mM Tris (pH 7.4), 10 mM iodoacetamide, 10 mM NaF, 1 mM sodium orthovanadate, and protease inhibitors (Sigma-Aldrich). Lysates were incubated at 4 °C for 15 min, followed by centrifugation at 13,000 × g for 15 min. The supernatant was preclarified with protein A/G-Sepharose beads (Amersham Biosciences) for 1 h at 4 °C, followed by the addition...
FIGURE 3. AICAR causes cell cycle arrest in S-phase. C6 glioma and U87MG astrocytoma cells were treated with AICAR at indicated concentrations. After overnight fixation cells were suspended in PBS with RNase A and propidium iodide and acquired for DNA content by flow cytometry using Modfit LT software. The first peak represents the cells in G₀/G₁ phase, the second peak with slashed bars represents the cells accumulated in S-phase, and the third peak represents cells in the M-phase. The data are also graphically represented as percentage of cells in S-phase and M-phase. The data are a representative of three separate experiments. ***, p < 0.001 compared with control; **, p < 0.01 compared with control.
of 1 μg/ml p85 mAb. After 2-h incubation at 4 °C, protein G-Sepharose beads were added, and the resulting mixture was further incubated for 1 h at 4 °C. The immunoprecipitates were washed twice with lysis buffer, once with PBS, once with 0.5 M LiCl and 100 mM Tris (pH 7.6), once in water, and once in kinase buffer (20 mM HEPES, pH 7.4, 5 mM MgCl₂, and 0.25 mM EDTA). PI3K activity was determined using a lipid mixture of 100 μl of 0.1 mg/ml phosphatidylinositol and 0.1 mg/ml phosphatidylserine dispersed by sonication in 20 mM HEPES (pH 7.0) and 1 mM EDTA. The reaction was initiated by the addition of 20 μCi of [γ-32P]ATP (3000 Ci/mmol, PerkinElmer Life Sciences) and 100 μM ATP, and terminated after 15 min by the addition of 80 μl of 1 N HCl and 200 μl of chloroform:methanol (1:1). Phospholipids were separated by TLC and visualized by exposure to iodine vapor and autoradiography (38).

**Statistical Analysis**—The data were statistically analyzed by performing the Student-Newman-Keuls Test.

**RESULTS**

**AICAR Inhibits Proliferation of Cancer Cells**—To investigate the effect of AICAR on the growth of various cancer cell lines, namely PC-3 (human prostate cancer cell), MCF-7 (human breast cancer cell line), C6 glioma (rat transformed brain glial cells), U87MG (human astrocytoma cell line), K-562 (human chronic myelogenous leukemia cells), and CEM (human T-lymphoblast cells), cells were plated in their respective
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A. C6 glioma and PC3 prostate cells were treated with AICAR at indicated concentrations and harvested at specified time points. Cell lysates were processed for the detection of phospho-AMPK (p-Thr-172) and phospho-ACC by immunoblot as discussed under "Materials and Methods." The blots are representatives of three individual experiments done. B, cell lysates were prepared from the vehicle- and AICAR-treated tumor tissues from two different sets of animals (each set had n = 6) and processed for the detection of phospho-AMPK (p-Thr-172) and phospho-ACC as above.

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medium for growth and treated with different concentrations of AICAR (0.25–1 mM) for 24 h, and cell proliferation was examined by [3H]thymidine uptake. AICAR inhibited the proliferation of all cell lines tested significantly in a dose-dependent manner (Fig. 1). All tested cell lines underwent significant proliferation inhibition, indicating that this phenomenon is widespread and not limited to a specific cell type/line. To further confirm this observation, a clonogenic assay was performed, where cells were treated with AICAR for 24 h, trypsinized and plated at a density of 300 cells/100-mm plate without AICAR. After 3 weeks, formed colonies were counted by staining the live cells with MTT. AICAR treatment significantly reduced the number of colonies being formed as compared with the untreated cells (Fig. 2), suggesting that a single treatment of AICAR treatment is sufficient to cause a sustained inhibition of proliferation in different cancer cell lines.

AICAR Causes Cell Cycle Arrest in S-phase—Inhibition in proliferation would indicate an anomaly in the cell cycle. To examine this, cells were treated with AICAR (0.5–1 mM), and phases of cell cycle were analyzed by flow cytometry. Treatment of cells with AICAR resulted in accumulation of cells in S-phase (peak with slashed bars), with almost no cells detected in M-phase (third peak) suggesting that inhibition in proliferation by AICAR is due to the arrest of cell cycle at S-phase (Fig. 3).

AICAR Inhibits Proliferation in Vivo—To investigate whether the anti-proliferative effects of AICAR extends to the in vivo system, we utilized the rat flank tumor model (34). Wherein, C6 glioma cells (1 × 10^6) were implanted aseptically in the right flank of the rat, and after 5 days of tumor formation, animals were treated with 100 mg/kg body weight of AICAR intraperitoneally. On day 14, animals were sacrificed, and the tumors were excised, weighed, and fixed. Weight of the tumors was taken as an index of tumor development and progression. AICAR treatment was able to reduce the growth of tumors in animals significantly (≥50%) when compared with untreated animals (Fig. 4A). To examine the status of proliferating cells in vivo, immunohistochemistry was performed on the sections of excised tumor tissues for PCNA (proliferating cell nuclear antigen), a marker for proliferating cells. AICAR significantly reduced the expression and number of cells exhibiting PCNA expression, indicating that the number of proliferating cells is reduced by AICAR treatment in vivo as demonstrated by immunohistochemistry and its expression by Western blot (Fig. 4, B and C). Thus, the anti-proliferative effect of AICAR is effective in vivo as well and can be exploited for applications in attenuating cancer cell growth.

AICAR Mediates Its Anti-proliferative Action via AMP-activated Protein Kinase—AICAR, is the earliest known AMPK activator, and most of its effects have been shown to be because of AMPK activation, although few reports of its AMPK-independent effects exist (22). To investigate if AMPK activation is responsible for the anti-proliferative effects observed by AICAR treatment, the phosphorylation of AMPK and its downstream target, ACC, an enzyme in the fatty acid synthesis pathway, was taken as an indicator of AMPK activation. AICAR induced the phosphorylation of AMPK and ACC in a dose- and time-dependent manner as demonstrated in C6 glioma and PC3 prostate cell lines (Fig. 5, A and B). Similar phosphorylation of ACC and AMPK was observed in vivo, in the AICAR-treated excised tumor tissue (Fig. 5C). Iodotubericidin is an inhibitor of adenosine kinase and inhibits the conversion of AICAR to its activated form ZMP inside the cell and thus inhibits activation of AMPK by AICAR. Cells were pretreated with iodotubericidin 30 min before the addition of AICAR (0.5–1 mM), and proliferation was measured after 16 h by [3H]thymidine uptake. Iodotubericidin treatment inhibited the proliferation arrest caused by AICAR thus indicating the involvement of AMPK (Fig. 6A). To further confirm the role of AMPK, C6 glioma cells were transiently transfected with dominant negative (DN) and constitutive active (CA) forms of AMPK along with eGFP expression vector. The cells were treated with AICAR for 18 h, and GFP-positive cells were analyzed by flow cytometry for DNA content to determine the cells in S-phase. C6 glioma cells transfected with AMPK dominant negative were not able to undergo S-phase arrest when treated with AICAR (Fig. 6B). Inversely, C6 glioma cells transfected with expression vector of constitutive active AMPKα1 were...
FIGURE 6. AICAR mediated its anti-proliferative action via AMPK. A, cells (CEM, K-526, and PC-3) were pretreated with iodotubericidin (0.1 μM) before addition of AICAR and assayed for [3H]thymidine incorporation. Iodotubericidin reversed the AICAR-induced proliferation block. The data are representative of three separate experiments performed in triplicates. ***, p < 0.001 compared with control; ###, p < 0.001 compared with AICAR (B and C) C6 glioma cells were transiently co-transfected with 2 μg of AMPK dominant negative (DN) (B) or AMPK constitutive active (CA) (C) and 1 μg of eGFP expression vector. The DNA content was normalized by pcDNA3. AICAR was added where indicated, and after 18 h cells were fixed overnight and analyzed for arrest in S-phase as detailed under "Materials and Methods." AICAR was not able to arrest the cells in the presence of AMPK DN (B), whereas AMPK CA expression was sufficient to arrest the cells in S-phase and showed additive effect with AICAR (C). The data are representative of three separate experiments. ***, p < 0.001 compared with control; ###, p < 0.001 compared with AICAR; ##, p < 0.01 compared with AICAR; #, p < 0.05 compared with AICAR; NS, non-significant compared with control.

D, cells were transfected with AMPK antisense (AS) and missense (MS) oligonucleotides along with eGFP expression vector and treated with AICAR. The level of AMPK protein was reduced by the transfection of AS, whereas MS had no effect (inset) at 72 h. The cells were fixed and processed for GFP-gated S-phase arrest. Antisense of AMPK abolished the AICAR-mediated S-phase arrest, whereas MS oligonucleotide had no effect. These data are representative of 3 separate experiments. ***, p < 0.001 compared with control; ###, p < 0.001 compared with AICAR; NS, non-significant compared with AICAR.
FIGURE 7. AICAR inhibits PI3K-Akt Pathway. A, C6 cells were treated with AICAR (1 mM) for 2 h and processed for PI3K activity as described under “Materials and Methods.” AICAR significantly reduced the PI3K activity as assessed by inositol 1,4,5-bisphosphate levels. The inositol 1,4,5-bisphosphate levels were measured by densitometry analysis. The blot is representative of three separate experiments. **, p < 0.01 compared with control. B, C6 cells were treated with AICAR for increasing time points as indicated, cells lysates were prepared and analyzed for Akt (Ser-473) and mTOR (Ser-1448) phosphorylation by Western blot as detailed under “Materials and Methods.” C, cell lysates were prepared from the treated tumor tissue from two different set of animals and processed for the detection of phospho-Akt, which was reduced by AICAR treatment.

FIGURE 8. AICAR regulates the expression of cdk inhibitors via AMPK. Protein expression of cell cycle inhibitors p21, p27, and p53 was increased by AICAR treatment as analyzed by immunoblot in C6 cells treated with AICAR (A) and tumor tissue (B). C, immunofluorescent microscopy images of tumor sections from vehicle and treated rats, stained with p21, p27, and p53 antibodies as described under “Materials and Methods.” D, C6 glioma cells were transiently transfected with 2 µg of AMPK dominant negative (DN) or AMPK constitutive active (CA) with DNA normalization done with pcDNA and treated with AICAR where indicated. Cell lysates were prepared and assessed by immunoblot for p21, p27, and phospho-Akt expressions. AICAR induced p21 and p27 expression and down-regulated phospho-Akt as before (lane 2), which was reversed by AMPK DN expression (lane 3) and had no effect with AICAR treatment (lane 4). AMPK CA α1 and α2 overexpression was able to induce the p21 and p27 expression and attenuate phospho-Akt by itself (lanes 5 and 6). The blots are representatives of three individual experiments done.
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found to be arrested at S-phase similar to AICAR-treated cells (Fig. 6C). Further, we used the antisense (AS) approach to knock out the expression of AMPK. C6 glioma cells were transfected with AMPK antisense (AS) and missense (MS) oligonucleotides, and levels of AMPK were observed 48 h post transfection. The level of AMPK protein was attenuated by AS, whereas MS had no effect (Fig. 6D, inset). Moreover, transfection with antisense of AMPK along with GFP expression vector in C6 glioma cells significantly reduced the AICAR-mediated S-phase arrest; however, MS oligonucleotide did not affect the potential of AICAR to arrest cells in S-phase (Fig. 6D). Taken together, these evidences point strongly toward a role for AMPK as an effective anti-proliferative system.

AICAR/AMPK Inhibits the PI3K-Akt Pathway—Because AICAR inhibits cell proliferation and PI3K-Akt is one of the most important pathways regulating proliferation, we examined the effect of AICAR on the PI3K-Akt pathway. C6 glioma cells were treated with AICAR (1 mM) for 2 h, and PI3K activity was assessed using phosphoinositol as a substrate, and we observed that AICAR treatment significantly reduced the PI3K activity (Fig. 7A). One of the downstream effectors of PI3K, Akt is the main mediator regulating proliferation (39). AICAR also reduced the phosphorylation of Akt in vitro and in vivo (Fig. 7Bii and C). It also inhibited the phosphorylation (Ser-1448) of mTOR (Fig. 7Bii), which is a downstream target of Akt and regulates protein synthesis and cell growth (40). Thus, attenuation of the PI3K-Akt pathway may be one of the mechanisms by which AMPK negatively regulates growth.

AICAR Regulates the Expression of Cyclin-dependent Kinase Inhibitors via AMPK—Because AICAR inhibits the cell proliferation by arresting cells at S-phase in vitro as well as in vivo, we examined the expression of cyclin-dependent kinase (cdk) inhibitors, which bind to cyclin-cdk complexes and inhibit the progression of cell cycle. AICAR induced the expression of p21 and p27, the cip/kip protein cdk inhibitors in a time-dependent manner (Fig. 8A). It also induced the expression of p53, which is known to regulate the cell cycle as well as p21 expression (41). The expression of p21, p27, and p53 proteins, were also increased in vivo, as assessed by immunohistochemistry of tissue sections and by Western blot analysis of protein isolated from excised tumor tissue (Fig. 8, B and C). The effect of AICAR on the expression of growth regulators is mediated via activation of AMPK, because transfected AMPK DN abolished the AICAR-mediated induction of p21, whereas the CA form of AMPKα1 and α2 induced the expression by itself (Fig. 8D). In case of p27, AMPK DN reduced the AICAR-induced expression, but AMPKα2 and α2 CA forms were only able to induce p27 protein marginally compared with AICAR. In case of Akt, DN AMPKα2-transfected cells did not respond to the AICAR-mediated inhibition in Akt phosphorylation, whereas, in CA-transfected cells, there was significant inhibition (Fig. 8E), indicating that AMPK activation is responsible for increase in cdk inhibitor protein expressions and inhibition of Akt phosphorylation.

LKB (AMPKK) Status Does Not Affect AICAR-mediated Growth Arrest—LKB is a recently discovered upstream target of AMPK (AMPK kinase, AMPKK), which phosphorylates AMPK at Thr-172 for its full activation. LKB itself is a tumor suppressor gene and inactivation of LKB results in predisposition to various cancers (27–29). It is being hypothesized that the anti-tumor effects of LKB are due to AMPK activity. To examine the possible involvement of LKB in AICAR/AMPK-induced growth arrest, we utilized LKB knock-out (LKB−−) and LKB WT (LKB+++/−) MEF cell lines. AICAR was able to induce the phosphorylation of AMPK and ACC to a similar extent in both MEFs (Fig. 9A). AICAR was able to inhibit proliferation in both knock-out and wild-type MEFs to a similar extent (Fig. 9B). This was further supported by similar S-phase arrest observed when C6 cells were transiently transfected with LKB dominant negative and wild-type expression vectors along with expression vectors of its cofactors, STRADα and MO25α, and treated with AICAR (Fig. 9C). These data indicate that AMPK activation by AICAR is sufficient to cause growth arrest and does not require activation by LKB.

DISCUSSION

In this study we have demonstrated that AMPK activation by AICAR results in growth arrest at S-phase due to inhibition of PI3K-Akt pathway and up-regulation of cdk inhibitors, independent of its upstream kinase LKB. This inference is based on the following observations: 1) Treatment of various cancer cell lines by AICAR attenuated the prolif-
induce their expression (Fig. 8). The induction of p27 by AMPK CA elevated expression of p21 and p27, whereas AMPK CA itself was able to mediated growth arrest, whereas AMPK CA expression was able to vector or AS oligonucleotide also resulted in the reversal of AICAR-

The growth arrest is mediated by inhibition of PI3K activity and Akt phosphorylation and up-regulation of cell-cycle inhibitor proteins p21, p27, and p53. 4) Activation of AMPK in the absence of LKB also results in growth arrest. We show here the direct relation between AMPK activation and growth inhibition in vitro and in vivo. In addition, these observations strongly indicate AICAR, an AMPK activator to be an efficient anti-proliferative agent in vitro and in vivo.

Activation of AMPK by AICAR, metformin, or thiazolidinediones or expression of constitutively active mutants has been shown to cause death or attenuate the growth of cancer cells. AICAR- and rosiglitazone-mediated AMPK activation caused proliferation block and cell death by inhibiting fatty acid and protein synthesis pathways and increasing p21 expression in prostate cells (22). Adenosine-induced AMPK was shown to cause apoptosis in gastric cancer cells (20), and activation of AMPK by AICAR and its CA form was shown to cause apoptosis in pancreatic cells by inducing JNK pathway (23). Similarly, AMPK induced JNK and caspase 3 activity resulting in apoptosis in liver cells (24). AMPK activation was also demonstrated to enhance H2O2-mediated apoptosis in neuroblastoma cells by inducing NF-κB and p38-JNK pathways (25). These studies, along with the present study, suggest AMPK as an efficient growth inhibitor and apoptosis inducer. On the other hand, it also has been shown to have a protective effect on stress-injured cells in heart ischemia and reperfusion injury model (15, 18). AMPK activation protects primary astrocytes from fatty acid-induced death by inhibiting de novo ceramide synthesis (17) and protects human umbilical vein endothelial cells from hyperglycemia by inhibition of caspase 3 and Akt activation (18) and by similar mechanism in thymocytes (19). In pancreatic cancer cells, AMPK was shown to bestow tolerance toward nutrient deprivation (43). These studies presented AMPK as a protective agent. The reason for these apparently opposing effects of AMPK activation in cell survival and cell death is not known, but it can be speculated that in actively dividing cancer cells, the inhibition of ATP-consuming processes by AMPK may be less compatible with their survival, whereas in non-dividing cells, the protective effects of AMPK have been observed under acute stress, the shutdown of ATP-consuming pathways may not alter the balance for survival.

Fig. 10. A schematic representation of effect of AICAR on proliferation of cancer cells. AICAR, upon entering the cell, is converted to ZMP, which activates AMPK. Upon activation AMPK increases the expression of p21, p27, and p53 proteins, which may be responsible for the S-phase arrest being observed. On the other hand it inhibits the PI3K activity and Akt phosphorylation, which results in inhibition of mTOR and speculative regulation of other targets like MDM2, Bad, and caspase 9, resulting in a proliferation and cell growth block. The overall signaling taking place results in a sustained proliferation arrest, which can ultimately lead to loss of viability due to onset of senescence or apoptotic pathways.
AMPK as a Potential Target for Treatment of Cancer

AMPK as a tumor suppressor system has gained more scientific interest because of its link with two tumor suppressors. One of these is the tuberous sclerosis complex 2 (TSC2), mutations of which cause tuberous sclerosis, an autosomal dominant disorder, which in humans is associated with hamartomatous polyps in multiple tissues and an increased risk of cancers. TSC2 forms an inhibitory complex with TSC1 and inhibits mTOR, leading to negative regulation of cell size and growth (31, 32). TSC2 is phosphorylated and inhibited by Akt. The observation that AMPK phosphorylates and activates TSC2 suggests yet another mechanism for the inhibitory effect of AMPK on growth (44, 45). In this regard our observation that AMPK inhibits PI3K activity and Akt phosphorylation (Fig. 7), points toward another route for regulating not only the TSC2-mTOR pathway but also other downstream players of Akt that control proliferation. The tumor suppressor, LKB1 (Stk11) functions as an upstream kinase, which phosphorylates and activates AMPK in physiological settings. Mutations of LKB1 have been found in Peutz-Jeghers syndrome, an autosomal dominant disorder characterized by multiple hamartomatous polyps (benign overgrowth of differentiated tissues) of the colon and a predisposition to cancers of the colon, pancreas, and other locations in the gastrointestinal tract (27, 29). Mutations of LKB1 typically occur in the catalytic domain, leading to loss of its kinase activity and presumably a failure to phosphorylate AMPK. According to our observation in LKB-null MEF cells, AICAR was able to activate AMPK and inhibit proliferation in both cells to a similar extent (Fig. 9), indicating that AMPK can act as a tumor suppressor even in absence of functional LKB. Thus, activation of AMPK by any other means or agents can also be exploited for the therapeutics for Peutz-Jeghers syndrome, where LKB is mutated.

Taken together, we conclude that activation of AMPK, by AICAR in this case results in activation of p21, p27, and p53, which results in cell cycle arrest. The elevation in the expression of p21 may be a direct effect of AMPK or may be occurring via p53, which remains to be explored. On the other hand it not only inhibits TSC2 complex as well documented by others (44, 45) but also attenuates the PI3K and Akt activity, which would further add to the negative regulation of TSC2-mTOR pathway (Fig. 10). Inhibition of Akt would also result in negative regulation of MDM2 that would help in up-regulating p53 activity, and forkhead, BAD, and caspase 9 (46), which would all assist in manifesting the anti-proliferative effect of AMPK activation. The exact mechanisms need to be worked out, because the regulation of proliferation and cell death by senescence or apoptosis. Overall, AMPK activation by AICAR or any other pharmacological agent is an attractive target for cancer therapy.

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REFERENCES

1. Hardie, D. G., and Carling, D. (1997) Eur. J. Biochem. 246, 259–273
2. Davies, S. P., Carling, D., and Hardie, D. G. (1998) Eur. J. Biochem. 186, 123–128
3. Kemp, B. E., Stapleton, D., Campbell, D. J., Chen, Z. P., Murphy, S., Walter, M., Gupta, A., Adams, J. J., Katsis, F., van Denderen, B., Jennings, I. G., Iseli, T., Michell, B. J., and Witters, L. A. (2003) Biochem. Soc. Trans. 31, 162–168
4. Minokoshi, Y., Kim, Y. B., Perouin, O. D., Fryer, L. G., Muller, C., Carling, D., and Kahn, B. B. (2002) Nature 415, 339–343
5. Yamasaki, T., Kemon, I., Minokoshi, Y., Ito, Y., Waki, H., Uchida, S., Yamashita, S., Noda, M., Kita, S., Ueki, K., Eto, K., Akamatsu, Y., Froguel, P., Foufelle, F., Ferre, P., Carling, D., Kimura, S., Nagai, R., Kahn, B. B., and Kadowaki, T. (2002) Nat. Med. 8, 1288–1295
6. Ruderman, N. B., Park, H., Kaushik, V. K., Dean, D., Constant, S., Prentki, M., and Saha, A. K. (2003) Acta Physiol. Scand. 178, 435–442
7. Kelly, M., Keller, C., Avilucea, P. R., Keller, P., Luo, Z., Xiang, X., Giralt, M., Hidalgo, J., Saha, A. K., Pederson, B. K., and Ruderman, N. B. (2004) Biochem. Biophys. Res. Commun. 320, 449–454
8. Luo, Z., Saha, A. K., Xiang, X., and Ruderman, N. B. (2005) Trends Pharmacol. Sci. 26, 69–76
9. Hong, S. P., Leiper, F. C., Woods, A., Carling, D., and Carlson, M. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 8839–8843
10. Woods, A., Johnstone, S. R., Dickerson, K., Leiper, F. C., Fryer, L. G., Neumann, D., Schlattner, U., Wallimann, T., Carlson, M., and Carling, D. (2005) Curr. Biol. 15, 2004–2008
11. Hawley, S. A., Boudaief, J., Reid, J. L., Mustard, K. J., Udd, L., Makela, T. P., Alessi, D. R., and Hardie, D. G. (2003) J. Biol. 2, 28
12. Fryer, L. G., Parbu-Patel, A., and Carling, D. (2002) J. Biol. Chem. 277, 25226–25232
13. Sullivan, J. E., Brocklehurst, K. J., Marley, A. E., Carey, F., Carling, D., and Beri, R. K. (1994) FEBS Lett. 353, 33–36
14. Corton, J. M., Gillespie, J. G., Hawley, S. A., and Hardie, D. G. (1995) Eur. J. Biochem. 239, 558–565
15. Russell, R. R., 3rd, Li, J., Coven, D. L., Hawley, S. A., Carling, D., and Hardie, D. G. (2004) Biochem. Biophys. Res. Commun. 321, 161–167
16. Boulton, D. A., Pan, D. A., and Hudson, E. R. (2003) Biochem. Biophys. Res. Commun. 304, 252–258
17. Hardie, D. G., Scott, J. W., Pan, D. A., and Hudson, E. R. (2003) Biochem. Biophys. Res. Commun. 304, 252–258
18. Chakrabarti, I., Hurley, P. T., Chi, J. H., Hall, J. S., Kaiser, M. G., and Bruce, J. N. (2000) Neurosurgery 47, 993–999; discussion 999–1000
19. Nath, N., Giri, S., Prasad, R., Singh, A. K., and Singh, I. (2004) J. Immunol. 172, 1273–1286
36. Giri, S., Nath, N., Smith, B., Viollet, B., Singh, A. K., and Singh, I. (2004) J. Neurosci. 24, 479–487
37. Culmsee, C., Monnig, J., Kemp, B. E., and Mattson, M. P. (2001) J. Mol. Neurosci. 17, 45–58
38. Giri, S., Rattan, R., Singh, A. K., and Singh, I. (2004) J. Immunol. 173, 5196–5208
39. Brazil, D. P., Yang, Z. Z., and Hemmings, B. A. (2004) Trends Biochem. Sci. 29, 233–242
40. Harrington, L. S., Findlay, G. M., and Lamb, R. F. (2005) Trends Biochem. Sci. 30, 35–42
41. el-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W., and Vogelstein, B. (1993) Cell 75, 817–825
42. Nath, N., Giri, S., Prasad, R., Salem, M. L., Singh, A. K., and Singh, I. (2005) J. Immunol. 175, 566–574
43. Kato, K., Ogura, T., Kishimoto, A., Minegishi, Y., Nakajima, N., Miyazaki, M., and Esumi, H. (2002) Oncogene 21, 6682–6690
44. Kimura, N., Tokunaga, C., Dalal, S., Richardson, C., Yoshino, K., Haru, K., Kemp, B. E., Witters, L. A., Miiura, O., and Yonezawa, K. (2003) Genes Cells 8, 65–79
45. Bolster, D. R., Crozier, S. J., Kimball, S. R., and Jefferson, L. S. (2002) J. Biol. Chem. 277, 23977–23980
46. Song, G., Ouyang, G., and Bao, S. (2005) J. Cell Mol. Med. 9, 59–71