

Full-length article

AICAR inhibits proliferation and induced S-phase arrest, and promotes apoptosis in CaSki cells¹

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Key words

AICAR; proliferation; apoptosis; CaSki cells

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Abstract

Aim: The aim of the present study was to determine the effect of 5-aminoimidazole-4-carboxamide-ribonucleoside (AICAR) on proliferation, cell cycle, and apoptosis in the human epithelial cervical cancer cell line CaSki cells. **Methods:** Cell count and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay were used to determine cell proliferation and viability. Hoechst 33258 staining was conducted to distinguish the apoptotic cells. Cell cycle and Annexin-V/propidium iodide staining were analyzed by fluorescence-activated cell sorting (FACS). A Western blot assay was used to evaluate the expression of AKT (also known as protein kinase B), mammalian target of rapamycin (mTOR), p53, and extracellular signal-regulated kinase (ERK). **Results:** AICAR (500 μ mol/L) significantly inhibited the proliferation of CaSki cells treated for 24, 48, and 72 h as determined by cell count. The cells at the G₁ and G₂ phases were dramatically decreased while cells at the S phase were increased in response to AICAR treatment for 24, 48, and 72 h. The MTT assay showed less viable cells and Hoechst fluorescent staining showed more apoptotic cells upon AICAR stimulation. The results of the Annexin-V staining demonstrated a time-dependent increase of apoptosis in cells treated with AICAR for 24, 36, and 48 h. Furthermore, AICAR activated caspase-3 in a time-dependent manner. It was also found that AICAR inhibited the phosphorylation of AKT and mTOR, which are important kinases regulating cell growth and survival. AICAR stimulation obviously increased the expression of the tumor suppressor p53 and the phosphorylation of ERK. **Conclusion:** AICAR inhibited proliferation and induced S phase arrest and promoted apoptosis in CaSki cells, which might be mediated by the downregulation of the AKT/mTOR pathway and the upregulation of the p53/ERK pathway.

Introduction

Cervical cancer is the second leading cause of cancer morbidity and mortality for women around the world, especially in many developing countries^[1]. The aberrancy of signaling pathways is critical in the pathogenesis and progression of cancers. Thus far, many signaling transduction components have been explored as potential therapeutic targets in cervical cancer, such as p53^[2], extracellular signal-regulated kinase (ERK)^[3], phosphatidylinositol 3-kinase

(PI3K)^[4], and mammalian target of rapamycin (mTOR)^[5].

5-Aminoimidazole-4-carboxamide-ribonucleoside (AICAR) is a well-known activator of the adenosine monophosphate (AMP)-activated protein kinase (AMPK), which has been proposed to act as a cellular fuel sensor^[6]. AMPK is activated when the cellular AMP/ATP ratio increases. Activated AMPK inactivates the ATP-consuming cellular events and activates ATP-generating processes. Recently, AMPK was reported to be involved in cell growth regulation and was identified as a potential target for cancer^[7]. It has a

close relationship with 2 tumor suppressor genes: LKB1 and tuberous sclerosis complex 2^[6], which are important negative regulators of cell size and growth. It was reported that AICAR could inhibit the proliferation of several cancer cell lines, such as C6 glioma cells, T98 G astrocytoma cells, MCF-7 breast cancer cells, PC-3 prostate carcinoma cells, and hematological cancer cells^[8,9]. AICAR could also upregulate the expression of tumor suppressor p53 in some of those cancer cell lines^[8] and can inhibit mTOR signaling^[10]. Recently, Nafz *et al* reported that AICAR could also induce human papillomaviruses (HPV) suppression in cervical cancer cell lines^[13].

The aim of the present study is to elucidate the role of AICAR in cervical cancer cells. We treated cervical cancer cell line CaSki with AICAR at different concentrations to dynamically observe the effect of AICAR on cell proliferation, cell cycle, and apoptosis. In addition, we also explored the effect of AICAR on some important kinases associated with growth control.

Materials and methods

Reagents Human cervical epithelial cell line CaSki was obtained from the American Type Culture Collection (Manassas, VA, USA). AICAR was obtained from Toronto Research Chemicals (Toronto, Canada). Fetal bovine serum (FBS) was from Hyclone (Logan, UT, USA). The Annexin-V kit was purchased from Biosea Biotechnology (Beijing, China). Phospho-ERK (phospho-Thr202/Tyr204), phospho-AKT (Ser473), AKT (also known as protein kinase B), and phospho-mTOR (Ser2448) were from Cell Signaling Technology (Beverly, MA, USA). The antibodies against proliferating cell nuclear antigen (PCNA), p53, caspase-3, ERK, and eukaryotic initiation factor 5 (eIF5) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture The CaSki cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin (Life Technologies, Gaithersburg, MD, USA). The cells were cultured at 37 °C in a humidified atmosphere with 5% CO₂.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay Exponentially growing cells were seeded at 1×10⁴ cells/well in 96-well plates and incubated with AICAR at various concentrations for 24, 48, and 72 h, respectively. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/mL; Sigma, St Louis, MO, USA) was added to each well and incubated for 4 h at 37 °C. The formazan product was dissolved by adding 150 µL DMSO to each well. The MTT absorbance value was detected at 490 nm

with a micro Sentence plate reader (Bio-Rad, Hercules, CA, USA).

Cell count assay The cells were plated at a density of 5×10⁴/well in 12-well plates in triplicate. DMEM supplemented with 10% FBS was used as the growth medium. After incubation with AICAR (500 µmol/L) for 24–72 h, the cells were harvested and exposed to 0.4% (w/v) trypan blue dye solution; the number of cells that were not stained with trypan blue was counted as viable cells.

Flow cytometry analysis For the cell cycle analysis, approximately 1×10⁶ cells were incubated with AICAR for 24–72 h. The cells were trypsinized and washed twice with cold phosphate-buffered saline (PBS) and then fixed in 70% ethanol at -4 °C overnight. Ethanol-fixed cells were resuspended in PBS containing 0.1 mg/mL RNase and incubated at 37 °C for 30 min. The pelleted cells were suspended in 0.5 mL of 50 µg/mL propidium iodide (PI) and analyzed with a flow cytometer (FACScan, Becton-Dickinson, San Jose, CA, USA). The cell cycle distribution was determined by ModFit LT cell cycle analysis software (Verity Software House, Topsham, ME, USA). For the detection of the percentage of apoptotic cell by Annexin-V/PI, after incubation with AICAR for 24–48 h, the CaSki cells were harvested and washed with cold PBS twice and then resuspended in 200 µL binding buffer at a concentration of 1×10⁶ cells/mL. The cells were incubated with 10 µL Annexin-V–fluorescein isothiocyanate (FITC) and 5 µL PI in the dark for 15 min. In total, 300 µL binding buffer was then added in each tube prior to being analyzed with the flow cytometer.

Analysis of nuclear morphology The cells were seeded in the 6-well plates and treated with AICAR (500 µmol/L) for 24–48 h. After washing twice with PBS, the cells were collected and fixed in methanol and then stained with 20 mg/mL Hoechst 33258 staining solution. Following washing with ice-cold PBS twice, the stained nuclei were examined and immediately photographed under a fluorescence microscope (Leica DMLB, Wetzlar, Germany). Apoptotic cells were defined on the basis of nuclear morphology changes, such as chromatin condensation and fragmentation.

Western blot analysis The CaSki cells were lysed in lysis buffer [20 mmol/L Tris-HCl (pH 7.4), 100 mmol/L NaCl, 10 mmol/L sodium pyrophosphate, 5 mmol/L EDTA, 50 mmol/L NaF, 1 mmol/L sodium vanadate, 0.1% SDS, 10% glycerol, 1% Triton X-100, 1% sodium deoxycholate, 1 µmol/L leupeptin, 0.1 µmol/L aprotinin, and 1 mmol/L phenylmethanesulfonyl fluoride (PMSF)]. The protein concentrations were measured by a BCA (Bicinchoninic Acid) protein assay kit (Pierce Biotechnology, Rockford, IL, USA). The samples (30 µg) were loaded and separated by SDS-PAGE and then electrophoretically

cally transferred to nitrocellulose membranes (Pall, Port Washington, NY, USA). The membranes were probed with various antibodies according to the supplier's protocol and visualized with peroxidase and an enhanced chemiluminescence system (Pierce Biotechnology, IL, USA).

Statistical analysis Data are expressed as mean±SEM. The statistical significance of the differences was determined by ANOVA followed by Student–Newman–Keuls (S–N–K) test or unpaired two-tailed *t*-tests. A value of $P < 0.05$ was considered significant.

Results

AICAR inhibits the proliferation of CaSki cells To determine the effect of AICAR on the proliferation in CaSki cells, the cells were incubated with AICAR at different concentrations (100, 250, 500, and 1000 $\mu\text{mol/L}$, respectively) for 24, 48, and 72 h. The number of cells was counted with a hemocytometer. After 24 h of incubation, all doses of AICAR significantly reduced the cell number (Figure 1A). The number of cells decreased from $(3.21 \pm 0.18) \times 10^4$ to $(2.23 \pm 0.19) \times 10^4$, $(2.29 \pm 0.31) \times 10^4$, $(1.67 \pm 0.06) \times 10^4$, and $(1.64 \pm 0.09) \times 10^4$, respectively. However, 100 $\mu\text{mol/L}$ AICAR did not significantly reduce the cell number when treated for 48 and 72 h. At 48 h, both 500 $\mu\text{mol/L}$ and 1000 $\mu\text{mol/L}$ AICAR significantly decreased the cell number compared with the control [from $(3.48 \pm 0.40) \times 10^4$ to $(1.31 \pm 0.39) \times 10^4$ and $(0.73 \pm 0.24) \times 10^4$] except for 250 $\mu\text{mol/L}$ AICAR which had no statistical effect on the reduction of the cell number (Figure 1B). After incubation for 72 h with AICAR, there was a dose-dependent decrease of the cell number for AICAR at 250, 500, and 1000 $\mu\text{mol/L}$ [from $(7.55 \pm 0.43) \times 10^4$ to $(5.47 \pm 0.15) \times 10^4$, $(1.19 \pm 0.21) \times 10^4$, and $(0.41 \pm 0.08) \times 10^4$; Figure 1C]. These results indicated that AICAR inhibited the proliferation of CaSki cells.

AICAR arrested cell cycle at S-phase The growth of mammalian cells is tightly controlled by cell cycles. To examine whether AICAR affects cell cycles, we stimulated CaSki cells with 500 $\mu\text{mol/L}$ AICAR and analyzed the cell cycles with the flow cytometer. AICAR significantly decreased the number of cells at both the G_1 and G_2 phases, but increased that of the cells at the S phase when measured at 24, 48, and 72 h (Figure 2A–2C). Therefore, the CaSki cells were arrested at the S phase by AICAR. We also measured the expression of PCNA, an important S-phase marker, by Western blotting. The results showed that the PCNA expression was obviously elevated in a time-dependent manner starting from 1 to 12 h after being normalized with internal control protein αIF5 (Figure 3). This further confirmed that the cells accumulated at the S phase.

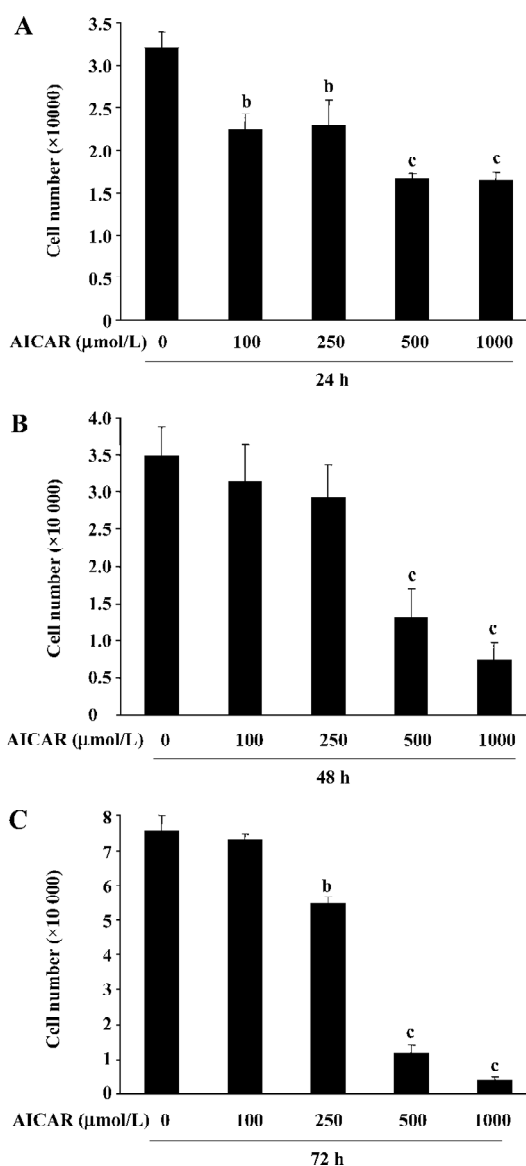


Figure 1. AICAR inhibits the proliferation of CaSki cells. CaSki cells were incubated with AICAR at concentrations of 100, 250, 500, and 1000 $\mu\text{mol/L}$, respectively, for (A) 24 h, (B) 48 h, and (C) 72 h. Then the cells were trypsinized for cell counts. Data shown are mean±SEM ($n=3$). ^b $P < 0.05$, ^c $P < 0.01$ compared with the control by S–N–K test.

AICAR induces apoptosis in CaSki cells The reduction of the cell number by AICAR in CaSki cells could also be due to the induction of apoptosis. We therefore examined whether AICAR would induce apoptosis by MTT assay, analysis of nuclear morphology, Annexin-V staining, and measurement of caspase-3 activation. As shown in Figure 4A, 500 $\mu\text{mol/L}$ AICAR time-dependently decreased the viability of CaSki cells from 24 to 72 h. The CaSki cells treated with AICAR showed less viable cells compared with the control, and

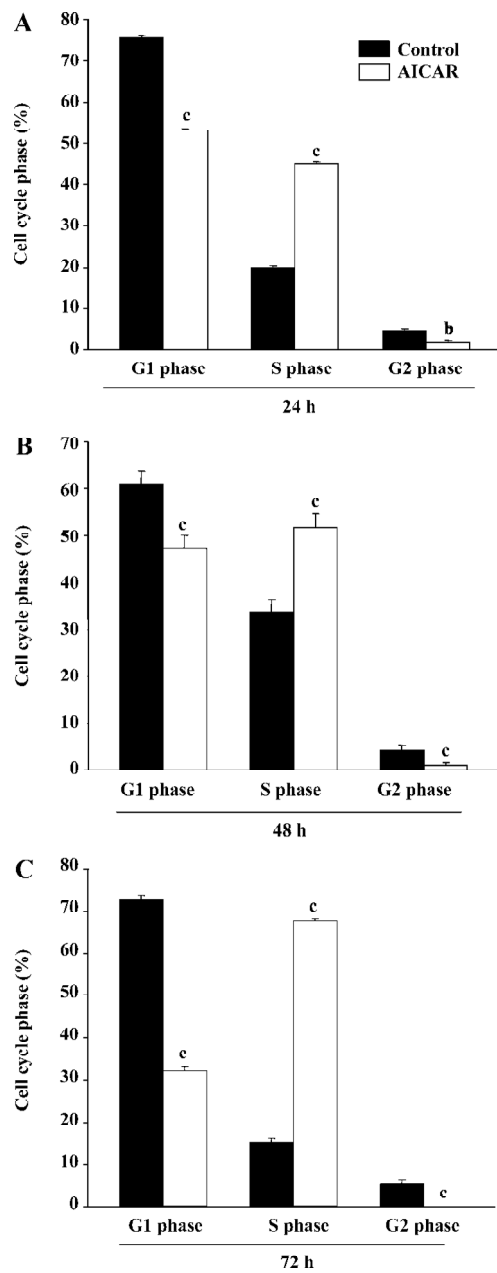


Figure 2. AICAR arrests CaSki cells at the S phase. After incubation with 500 $\mu\text{mol/L}$ AICAR for (A) 24 h, (B) 48 h, and (C) 72 h, the CaSki cells were fixed, stained with PI, and analyzed for DNA content by flow cytometry. Data were obtained from 15 000 events and were represented as a percentage of cells in the G₁ phase, S phase, and G₂ phase. ^b $P < 0.05$, ^c $P < 0.01$ compared with the control ($n = 3$).

showed more apoptotic cells which appeared as membrane blebbing, cell condensation, chromatin condensation, and formation of an apoptotic body (Figure 4B). One of the key features in apoptotic cells is the translocation of phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane. Annexin-V has a strong affinity for PS and thus

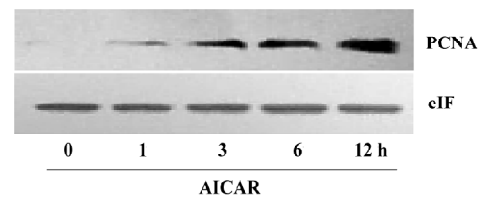


Figure 3. AICAR treatment upregulates PCNA expression. CaSki cells were challenged with AICAR for different hours as indicated, and cell lysates were collected to immunoblot with the anti-PCNA antibody. Membrane was stripped and reprobed with the internal control anti-eIF5 antibody. Experiments were repeated 3 times with comparable results.

can measure apoptosis. As shown in Figure 4C, incubation with AICAR increased the Annexin-positive cells from $4.2\% \pm 0.93\%$ to $9.36\% \pm 0.41\%$, $12.78\% \pm 0.80\%$, and $27.59\% \pm 1.17\%$ at 24, 36, and 48 h, respectively. Furthermore, the cleaved activated form of caspase-3 was detected and time-dependently increased in the cells treated with AICAR for 24, 36, and 48 h (Figure 4D). Taken together, these results suggested that AICAR not only inhibited proliferation, but also induced apoptosis in CaSki cells.

AICAR suppresses the AKT/mTOR pathway and enhances the p53/ERK pathway

The PI3K/AKT signaling pathway is crucial to many aspects of cell growth, survival, and apoptosis^[11]. The constitutive activation of AKT has been involved in the pathogenesis and progression of cervical cancer^[12]. Under basal level, AKT was constitutively phosphorylated in CaSki cells (Figure 5A). AICAR did not change the phosphorylation level of AKT when treated for 6 h, but the phosphorylation of AKT was time dependently inhibited by AICAR treatment between 12 and 48 h. We also measured the activation of mTOR, which is one of the important substrates of AKT. It showed a similar pattern of inhibition like AKT. An obvious inhibition of mTOR by AICAR was observed at 12 h (Figure 5A). These results indicated that AICAR may inhibit proliferation and induce apoptosis partially by the repression of the AKT/mTOR pathway. p53 plays a crucial role in the proliferation and survival of cancer cells. At basal level, the p53 protein expressed was quite low, but treatment with AICAR could time-dependently elevate the p53 protein expression following 3 h of stimulation (Figure 5B). We also examined the phosphorylated status of ERK, which was identified as an important mediator of apoptosis in CaSki cells downstream of p53^[3]. Our results showed that the phosphorylated ERK at basal level could not be detected in our system and it remained invisible within 12 h after incubation with AICAR.

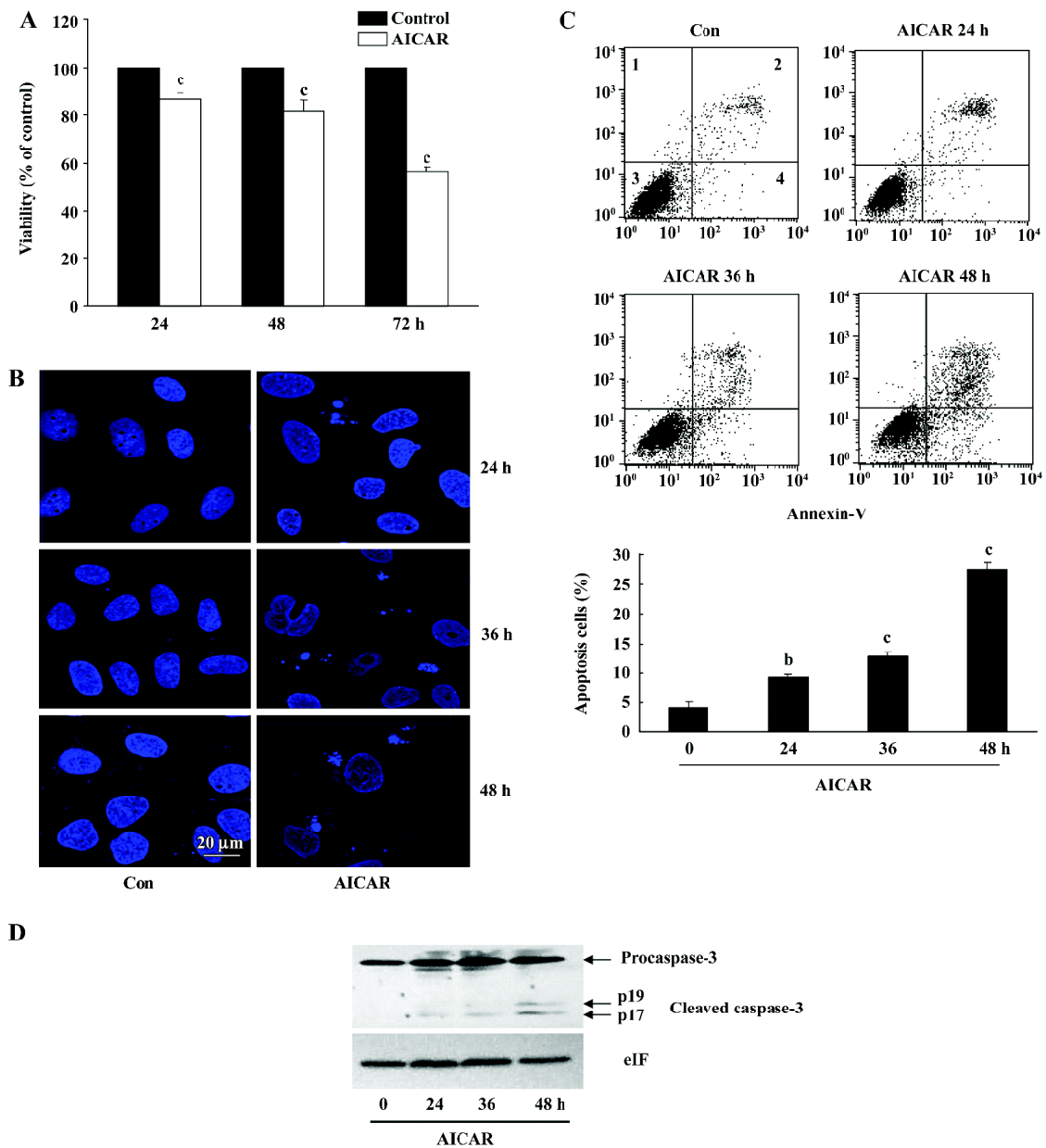


Figure 4. AICAR induces apoptosis in CaSki cells. (A) cells were treated with 500 μmol/L AICAR for 24, 48, and 72 h, respectively. Cell viability was assayed by MTT and data were expressed as a percentage of the control (mean±SEM). ^c*P*<0.01 compared with each corresponding control. (B) after stimulation with 500 μmol/L AICAR for 24, 36, and 48 h, respectively, the CaSki cells were stained with Hoechst 33258 and observed under a fluorescence microscope. (C) after stimulation with 500 μmol/L AICAR for 24–48 h, the cells were stained with FITC-conjugated Annexin-V/PI. Percentage of Annexin-V/PI-stained cells was determined by flow cytometry. Dot plot was divided into a quadrant: (1) necrotic cells, (2) late apoptotic cells, (3) living cells, and (4) early apoptotic cells. Graphs demonstrate the percentage of cells in early and late apoptosis. This is a representative of 3 separate experiments. Values are expressed as mean±SEM (^b*P*<0.05, ^c*P*<0.01 compared with the control using the S-N-K test). (D) after treatment with 500 μmol/L AICAR for different times as indicated, the cell lysates were separated with SDS-PAGE and hybridized with an anticaspase 3 antibody and anti-eIF5 antibody.

Intriguingly, AICAR increased ERK phosphorylation at 24 h, and further enhanced the phosphorylation at 36 and 48 h (Figure 5C). Taken together, these results demonstrated that AICAR inhibited the AKT/mTOR pathway and potentiated

the p53/ERK pathway, which may be responsible for the inhibition of AICAR on proliferation and the induction of apoptosis.

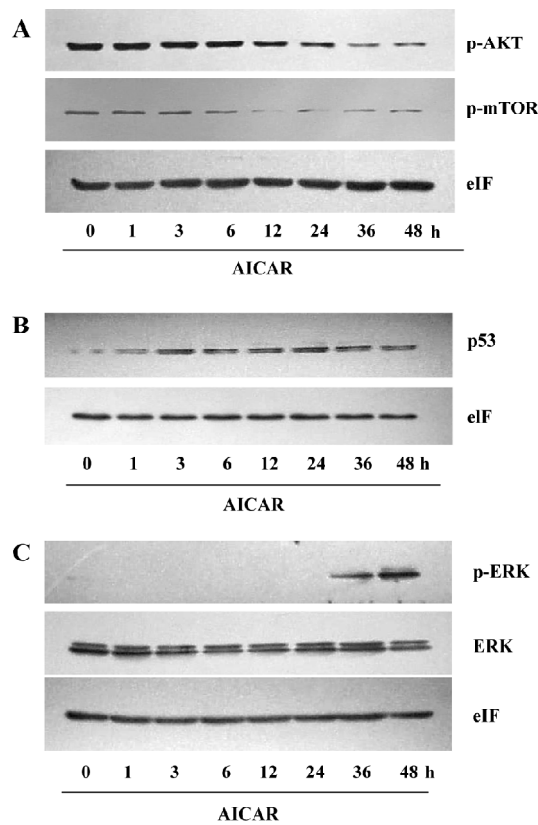


Figure 5. AICAR repressed the AKT/mTOR pathway, but enhanced the p53/ERK pathway. CaSki cells were incubated with AICAR (500 $\mu\text{mol/L}$) for different times as indicated, and cell lysates were immunoblotted with an (A) anti-phospho-AKT antibody, an anti-phospho-mTOR antibody, and an anti-eIF5 antibody; (B) an anti-p53 antibody and anti-eIF5 antibody; and (C) anti-phospho-ERK antibody, an anti-ERK antibody, and an anti-eIF5 antibody. Membranes were also stripped and reprobed with an anti-eIF5 antibody. All of the results were repeated 3 times.

Discussion

In the present study, we found that AICAR inhibited proliferation and induced apoptosis in CaSki cells. This is the first time that it has been demonstrated that AICAR arrests the cell cycle of CaSki cells at the S phase. It was found that AICAR inhibited the AKT/mTOR pathway and upregulated the p53/ERK pathway, which was probably associated with the inhibition of proliferation and the induction of apoptosis by AICAR.

As a cellular energy sensor, AMPK has recently been found to be an important regulator of cell growth, proliferation, and apoptosis^[6]. AICAR is an extensively used activator of AMPK. It is transported inside the cells through the adenosine transporter and phosphorylated by adenosine kinase to form zeatin riboside-5-monophosphate, which

activates AMPK by mimicking AMP. The activation of AMPK with AICAR significantly inhibited the proliferation of CaSki cells as indicated by the results from the cell count and MTT assay. This observation is consistent with a previous study in other cells^[8]. Interestingly, the lower dose of AICAR at a concentration of 100 $\mu\text{mol/L}$ showed an obvious inhibition of proliferation at 24 h, but had no effect at 48 and 72 h. AICAR at 250 $\mu\text{mol/L}$ could significantly reduce the cell number at 24 and 72 h but not at 48 h. This may indicate that cervical cancer cells exhibit different sensitivity to AMPK activation at different times, and it is also possible that these doses induced an approximately equivalent counteracting force of proliferation in the cells, resulting in the fluctuation of cell numbers. Further studies are still needed to elucidate this paradox. Our results demonstrated that 500 $\mu\text{mol/L}$ AICAR had already exerted a strong suppression on proliferation, and this concentration was much lower than that used in other cells which ranged from 1 and 5 mmol/L ^[13]. Cell proliferation is regulated by cycle machinery, which are balanced by cyclin-dependent kinases (CDK), CDKinhibitors (CDKI), and tumor suppressors^[6]. In some cancer cells, AICAR could upregulate the expression of CDKI, like P21 and p27, and elevate the level of p53, leading to cell cycle arrest at the G₁ phase^[8]. Interestingly, the effect of AICAR on CaSki cells is quite different. AICAR decreased the number of cells at the G₁ and G₂ phases and increased the number of cells at the S phase, resulting in an attenuation of cells entering the G₂ phase (Figure 2). Thus, PCNA, an index of the S phase, was also upregulated by AICAR (Figure 3). The entry of the G₂ phase is mainly regulated by cyclin A and cyclin B^[14], and it remains unknown as to whether AICAR affects these 2 cyclins.

The reduction of the cell number may also be due to apoptosis or cell death. The results of the cell viability showed an obvious decrease of living cells in the AICAR-treated group (Figure 4A). CaSki cells with AICAR stimulation also had typical morphological changes of apoptosis (Figure 4B). Furthermore, AICAR increased Annexin-V positive cells and activated caspase-3, a key effector of the apoptotic signaling pathways (Figure 4C, 4D). These results indicated that AICAR not only inhibited proliferation, but induced apoptosis in cervical cancer cells as well. The PI3K pathway plays an important role in cervical cancer^[4]. As a cardinal downstream effector, AKT was time dependently dephosphorylated by AICAR (Figure 5A). The effect of AICAR on AKT phosphorylation varies for different cell types. In vessel smooth muscle cells^[15] and lung cancer cells^[16], there was no change of AKT phosphorylation by AICAR, while AICAR could inhibit AKT phosphorylation in

several cancer cell lines^[8]. mTOR is an important substrate of AKT and we found that AICAR inhibited mTOR phosphorylation (Figure 5A), which further supports the suppressive effect of AICAR on the PI3K pathway. Rapamycin, a mTOR inhibitor, now has been used clinically as an anti-cancer drug^[17]. Our observation suggests that AICAR may be an important potential drug in cervical cancer treatment.

Most cervical cancer cases are found to be infected with HPV, and the viral E6 protein forms a ternary complex with the cellular E6-AP protein, which expedites the degradation of p53 in these cells. Therefore, p53 expression is relative lower in cervical cancer cells. We found AICAR could upregulate p53 expression (Figure 5B). This result is consistent with some previous reports in other cell types^[8]. As an important tumor suppressor, p53 could regulate CDK, CDKI, and many other substrates to regulate cell growth. A recent study suggested that ERK activation were also important in DNA damage-induced apoptosis^[18]. Singh *et al*^[3] reported that in SiHa and CaSki cells ERK activation was required in carboplatin-induced apoptosis, and p53 regulated the activation of ERK. In agreement with this new pathway, our results showed that AICAR apparently elevated the phosphorylation of ERK (Figure 5C).

In conclusion, we found that AICAR inhibited proliferation and induced apoptosis in cervical cancer cells, which suggests that AMPK may be an important potential target for cervical cancer treatment and AICAR may be a useful therapeutic drug against cervical cancer.

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