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Biochemical and Biophysical Research Communications 321 (2004) 161–167
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AMP-activated protein kinase activators can inhibit the growth of prostate cancer cells by multiple mechanisms

Xiaoqin Xiang [a], Asish K. Saha [a], Rong Wen [b], Neil B. Ruderman [a], Zhijun Luo [a,*]

[a] *Diabetes Research Unit, Section of Endocrinology, Department of Medicine, Boston University School of Medicine, Boston, MA 02118, USA*
[b] *Department of Ophthalmology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, USA*

Received 24 June 2004

Abstract

Prostate cancer cells require high rates of de novo fatty acid synthesis and protein synthesis for their rapid growth. We report here that the growth of these cells is markedly diminished by incubation with activators of AMP-activated protein kinase (AMPK), a fuel-sensing enzyme that has been shown to diminish both of these processes in intact tissues. Inhibition of cell growth was observed when AMPK was activated by either 5-aminoimidazole-4-carboxamide riboside (AICAR) or the thiazolidinedione rosiglitazone. Thus, a 90% inhibition of the growth of androgen-independent (DU145, PC3) and androgen-sensitive (LNCaP) cells was achieved after 4 days of exposure to one or both of these agents. Where studied, this was associated with a decrease in the concentration of malonyl CoA, an intermediate of de novo fatty acid synthesis, and an increase in expression of the cell cycle inhibitor p21. In addition, AICAR inhibited two key enzymes involved in protein synthesis, mTOR and p70S6K, and blocked the ability of the androgen R1881 to increase cell growth and the expression of two enzymes for de novo fatty acid synthesis, acetyl CoA carboxylase and fatty acid synthase, in the LNCaP cells. The results suggest that AMPK is a potential target for the treatment of prostate cancer.

Keywords: AMP-activated protein kinase; Prostate cancer cell; Growth; Survival; Acetyl CoA carboxylase; Fatty acid synthase; mTOR; p70S6K; p21

Many studies have demonstrated the association of altered fatty acid metabolism with prostate neoplasia [1]. Thus, while the rate of de novo fatty acid synthesis is very low in normal tissues of an adult, it is remarkably increased in cells derived from prostate, breast, and colon cancers, accounting for over 90% of the fatty acid in their glycerolipids. In keeping with this, fatty acid synthase (FAS) is highly expressed in almost all prostate cancers so far tested [2–8]. The expression of FAS varies with the level of androgen at an early stage in many prostate cancers, and it is upregulated during androgen-independent progression when the cells become more malignant. Swinnen et al. [9] have shown that androgen induces the expression and proteolytic maturation of the transcription factors SREBP-1 and -2. These transcriptional regulators bind to the sterol regulatory element on genes encoding FAS, and other enzymes of the de novo fatty acid synthesis pathway including ATP citrate lyase, acetyl CoA carboxylase (ACC), and malic enzyme and increase their expression. Schrijver et al. [10] have employed the RNA interference technique to provide direct evidence that FAS is required for the growth of prostate cancer cells.

An enzyme that has been shown to inhibit fatty acid synthesis and the activities of some of the key enzymes that regulate it is the AMP-activated protein kinase (AMPK). AMPK belongs to a family of serine/threonine protein kinases that is highly conserved from yeast to mammals. It is a heterotrimer containing a catalytic subunit (α) and two regulatory subunits (β and γ) [11]. In mammalian tissues, AMPK is activated by such hormones as leptin and adiponectin which are secreted by adipocytes, and by stresses such as hypoxia, ischemia,

* Corresponding author. Fax: 1-617-638-7094.
E-mail address: zluo@bumc.bu.edu (Z. Luo).

0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.06.133

glucose deprivation, and in some tissues, most notably skeletal muscle, by exercise. In many of these studies AMPK activation followed a decrease in the energy state of the cells, as reflected by an increase in the AMP/ATP ratio [12,13]. Enhanced binding of AMP to the γ-subunit of AMPK facilitates the phosphorylation of threonine 172 on the activation loop of the α-subunit by upstream AMPK-kinase(s) (AMPKK) [14]. AMPK activation by this mechanism helps to maintain the viability of stressed cells by enhancing processes that increase ATP generation (e.g., fatty acid oxidation and in a few tissues such as heart and kidney, glycolysis), and by inhibiting others that consume ATP and are not immediately required for survival. The latter include fatty acid, cholesterol and protein synthesis [13].

The compound, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) was the first pharmacological agent shown to activate AMPK. It is cell permeable and is converted to ZMP, an analogue of AMP, by an adenosine kinase [15]. Considerable information about the role of AMPK in cellular metabolism has been obtained with AICAR, although this agent does have actions that are not mediated by AMPK. Recently, several pharmacological agents that are used in the treatment of type 2 diabetes, including thiazolidinediones (TZDs), and metformin, an agent that inhibits hepatic glucose production, have also been shown to activate AMPK, although the mechanism of their action is incompletely understood. When activated, AMPK phosphorylates and inhibits ACC, and diminishes glucose-induced expression of the genes for ACC, FAS, and other enzymes by inhibiting the transcription factors SREBP1C and CHREBP1C [16–18]. In addition, AMPK has been shown to diminish protein synthesis by activating TSC2 leading to inhibition of mTOR and subsequent to this p70S6 kinase [19]. Since many of these factors are upregulated in prostate cancer, AMPK could be a target its therapy. The present study represents a first step in testing this possibility.

Materials and methods

Reagents. 5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranoside was purchased from Toronto Research Chemicals (Ont., Canada) and the synthetic androgen R1881 was from Sigma (St. Louis, MO). Rosiglitazone was a gift from GlaxoSmithkline (Research Triangle Park, NC). Antibodies against AMPK, ACC, mTOR, and p70S6K, and their phosphorylated forms were obtained from Cell Signaling (Beverly, MA). The monoclonal antibodies for p21 and fatty acid synthase were from BD Biosciences (San Diego, CA). Fetal bovine serum (FBS) and charcoal/dextran-treated FBS (CT-FBS) were purchased from Hyclone (Logan, UT) and cell culture media were from Gibco-BRL (Grand Island, NY).

Cell culture and treatment. Human prostate cancer PC3 cells were grown in F-12 media supplemented with 10% FBS and 2 mM L-glutamine, at 37 °C, under 5% CO_2. LNCaP cells were grown in RPMI1640 supplemented with 10% FBS under the same conditions. AICAR or rosiglitazone was added to the culture media as indicated. To determine the effect of AICAR on androgen action, the LNCaP cells were switched to an RPMI1640 medium containing 5% or 1% CT-FBS in the presence or absence of R1881 for 24 h and AICAR as indicated. Viable cells were counted by light microscopy after trypsinization and staining with 0.2% trypan blue.

Western blot analysis. Cells were washed with phosphate-buffered saline (PBS) and lysed with a buffer containing 25 mM Tris–HCl, pH 7.8, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4, 25 mM β-glycerolphosphate, 4 mM $Na_4P_2O_7$, 1 mM DTT, 1% NP-40, and protease inhibitors. Cell debris was removed by centrifugation at 14,000*g* at 4 °C for 15 min and the protein concentration of the supernatant was determined with a Bio-Rad protein assay kit. Cell extracts were resolved by SDS–PAGE and electrophoretically transferred to polyvinylidene difluoride membranes (Millipore). The membranes were blocked with milk and subsequently incubated with specific antibodies and a horseradish peroxidase-conjugated second antibody. Immunoreactive bands were visualized by the enhanced chemiluminescence.

Malonyl CoA assay. Malonyl CoA was radioisotopically assayed according to McGarry et al. [20], as modified by our laboratory [21].

Results

Effects of AICAR on the growth/viability of androgen-independent PC3 and Dul45 cells

Prostate cancer cells were incubated with AICAR and the number of attached cells was counted. As shown in Fig. 1A, after a 4-day incubation with AICAR, the net expansion of viable cells (i.e., cell growth) was less than that of untreated cells (IC_{50} = 40–50 μM). The effect of a maximally effective concentration of AICAR (100 μM) on the growth of PC3 cells after various incubation times is shown in Fig. 1B. The number of cells was slightly less on day 1 following treatment with AICAR than in its absence, but it was still greater than on day 0, indicating that proliferation was occurring. Thereafter, an increasing fraction of cells exposed to AICAR started to detach and by day 4, the total cell number was less than on day 0. These effects of AICAR were closely paralleled by AMPK activation, as indicated by an increase in the phosphorylation of AMPK at T172 in the absence of an increase in AMPK abundance. Induction of the cell cycle inhibitor p21 was also observed.

To ascertain whether the inhibition of cell growth caused by AICAR is reproduced when another AMPK activator is used, PC3 cells were incubated with rosiglitazone, an agent recently shown to activate AMPK in cultured cells [22]. After 4 days of treatment, AICAR (100 μM) decreased cell number by 80% and rosiglitazone (20 μM) by 60%. Both agents decreased the concentration of malonyl CoA by 40–50% (Fig. 2), strongly suggesting that they had activated AMPK and inhibited ACC.

Effects of AICAR on androgen-dependent LNCaP cells

We next examined the effect of AICAR on the growth of the androgen-sensitive human prostate adenocarcinoma

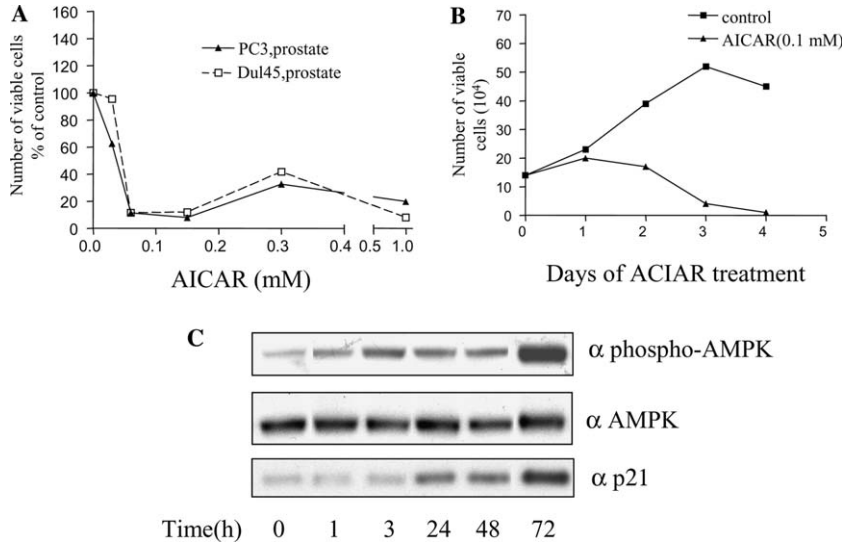

Fig. 1. AICAR inhibition of cancer cell growth. (A) PC3 and Du145 prostate cancer cells, as indicated, were seeded onto 6-well plates in their specific culture media containing a high concentration of glucose. AICAR was added the next day at the indicated doses. After 4 days, cells were counted with a hemacytometer. The results are expressed as percent of control cells grown in the absence of AICAR. (B) PC3 cells (1.2×10^5) were plated on 6-well plates and on the next day vehicle or AICAR was added to the culture medium and viable cells were counted at different times, as indicated. (C) Extracts (20 μg) of PC3 cells treated with AICAR (100 μM) were blotted with antibodies against phospho-AMPK (T172), AMPK (α1 + α2 catalytic subunit isoforms), and p21.

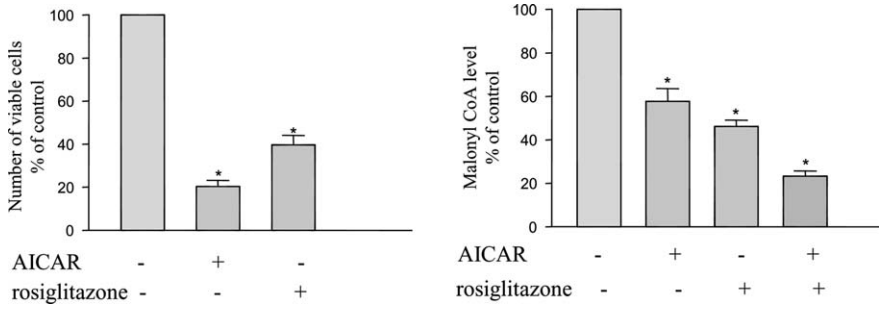

Fig. 2. Effects of AICAR and rosiglitazone on cell growth and malonyl CoA levels. PC3 cells were grown in the presence or absence of AICAR (100 μM) or rosiglitazone (20 μM). After 4 days, cells were counted (left) and the concentration of malonyl CoA relative to cellular protein was assayed in cell extracts (right). The results are expressed as percent of the values of the control cells cultured in the absence of these agents (means ± SEM, $n = 3$). *Result of one-way ANOVA test ($P < 0.001$).

LNCaP cell line, which possesses many of the properties of human prostate cancers [23]. An initial examination of the dose effect of AICAR showed an inhibition of the growth of the LNCaP cells similar to that of PC3 cells ($IC_{50} \sim 50$ μM) (Fig. 3A). After 4 days, the total number of LNCaP when grown in the presence of AICAR was less than that at its starting point (1.6×10^5 cells/well vs 5×10^5 cells/well) (Fig. 3A). Incubation with the synthetic androgen R1881 for 4 days increased the growth of LNCaP cells by nearly twofold, an effect prevented by AICAR (Fig. 3B). On the other hand, AICAR caused an even greater reduction in cell growth in the absence of R1881, suggesting that the androgen conferred some protection against its inhibitory action.

AICAR inhibits the stimulation of fatty acid and protein synthesis by R1881

To explore the mechanism underlying the inhibition of androgen-stimulated growth by AICAR, we first assessed its effect on key enzymes of fatty acid synthesis. For this purpose, LNCaP cells were cultured in 5% CT-FBS for two days and then switched to 1% CT-FBS to minimize the effects of other factors in the serum. On the second day, R1881 was added to the medium. As shown in Fig. 4A, the androgen increased the concentration of malonyl CoA in a time-dependent manner, resulting in a nearly twofold increment by 24 h (Fig. 4A). This stimulatory effect of R1881 was attenuated by the addition of AICAR (Fig. 4B).

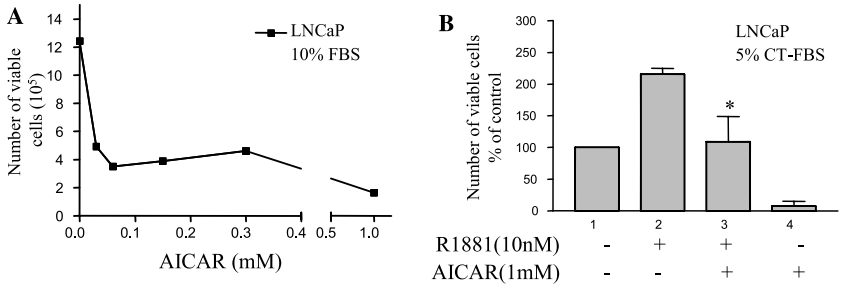

Fig. 3. Effects of AICAR on the growth of LNCaP cells. (A) Cells (5×10^5 cells/well) were cultured in the presence or absence of AICAR at the indicated concentrations for 4 days and then counted. (B) Cells were grown in 5% CT-FBS with or without additives for 4 days and then counted. Results are expressed as percent of the number of cells grown in media containing neither R1881 nor AICAR (means ± SEM, $n = 3$). *$P < 0.001$ (columns 3 vs 2).

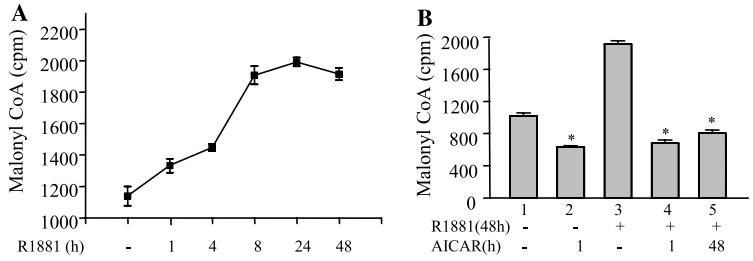

Fig. 4. Inhibition of androgen-induced malonyl CoA production by AICAR. (A) LNCaP cells were cultured in 5% CT-FBS medium for two days and then switched to 1% CT-FBS. After 24 h, R1881 (10 nM) was added and the incubations were continued for 1–48 h, as indicated. Malonyl CoA was assayed radio-enzymatically. (B) R1881 or vehicle was included in the culture medium (1% CT-FBS) for 48 h. AICAR (100 μM) was either added 1 h prior to the end of the incubation or it was present for the entire 48 h. Results of malonyl CoA assay are expressed as cpm incorporated into fatty acids (means ± SEM, $n = 3$). *$P < 0.001$ (2 vs 1; 4, 5 vs 3).

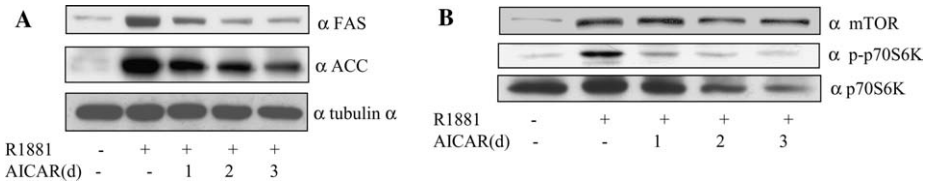

Fig. 5. AICAR inhibits key enzymes for fatty acid and protein synthesis. LNCaP cells were cultured in 5% CT-FBS medium for two days and then switched to 1% CT-FBS in the presence or absence of the R1881 (10 nM). Twenty-four hours later, AICAR (100 μM) was added to the medium and the cells were incubated for 1, 2 or 3 days as indicated. (A) Cell extracts were blotted with antibodies against FAS (1 μg extract) and ACC (20 μg extract) and tubulin α (20 μg extract). (B) Cell extracts, the same as A, were blotted, with antibodies against mTOR, phosphorylated (S389), and non-phosphorylated forms of p70S6K, respectively. Results are a representative of four independent experiments.

Consistent with previous reports [9,24], we also found that the androgen upregulated the expression of both FAS and ACC protein (Fig. 5A). This too was progressively inhibited by AICAR.

We next examined the effect of AICAR on mTOR and p70S6K in LNCaP cells. Despite the fact that PI3K pathway is constitutively active in these cells due to a loss-of-function mutation of the *PTEN* gene [25], when the cells were serum starved, the expression of mTOR was decreased (data not shown). As shown in Fig. 5B, R1881 induced the expression of mTOR in LNCaP cells, leading to an increase in phosphorylation of p70S6K. Incubation with AICAR did not significantly affect the abundance of mTOR; however, it dramatically decreased both the abundance of p70S6 kinase and its phosphorylation by mTOR. A similar inhibition of mTOR/p70S6K was found in PC3 cells with a loss-of-function mutation of the *PTEN* gene [25] (data not shown).

Discussion

In light of the inhibitory effect of AMPK on fatty acid synthesis, we hypothesized that sustained activation of AMPK would diminish the viability of cancer cells that are heavily dependent on de novo fatty acid synthesis for their growth. In keeping with this notion, in the present study we found that two pharmacological AMPK activators, AICAR and rosiglitazone, diminished the

viability of several cultured prostate cancer cell lines. Although more definitive studies are needed to prove that the effects of these agents are mediated by AMPK, the correlation between their effects on cell viability and AMPK activation, decreases in malonyl CoA, and where studied (AICAR only), inhibition of androgen-induced expression of FAS and ACC, are consistent with this notion. Also, we found that AICAR inhibited mTOR and p70S6K, another reported effect of AMPK activation [26].

A novel finding was that AICAR increased the expression of the cell cycle inhibitor p21. In a recent study, Immaura et al. [27] reported that AICAR, by activating AMPK, suppresses the growth of the hepatocellular carcinoma HepG2 cells, an effect attributed to the accumulation and phosphorylation of the tumor suppressor p53, and secondarily the induction of p21. Since PC3 cells do not contain wild-type p53 alleles [28], the induction of p21 by AICAR observed in the present study was probably p53-independent. Collectively, these findings suggest that AMPK activation can inhibit the growth of prostate cancer cells by at least three distinct mechanisms. Whether, because of these multiple actions, prostate cancer cells are especially sensitive to therapies that activate AMPK will require comparisons with other cancer and non-cancer cell studies under similar conditions.

AMPK has recently been linked to two tumor suppressors. One of them, LKB1 (also referred to as Stk11), functions as an upstream kinase that phosphorylates and activates AMPK in physiological settings [29,30]. Mutations of LKB1 have been found in patients with Peutz–Jeghers syndrome (PJS), 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 [31]. Mutations of LKB1 typically occur in the catalytic domain, leading to a loss of its kinase activity and presumably a failure to phosphorylate AMPK. Since LKB1 phosphorylates at least 13 other targets, it remains unclear whether AMPK activation accounts for its anti-tumor action [32].

A second tumor suppressor that has been linked to AMPK is the tuberous sclerosis complex 2 (TSC2). TCS2 forms an inhibitory complex with TSC1 that binds to and inhibits mTOR, leading to negative regulation of cell size and growth [33]. Mutations of TSC1 and TSC2 cause tuberous sclerosis, an autosomal dominant disorder [34], which in humans is associated with hamartomatous polyps in multiple tissues and an increased risk of cancers. TSC2 is phosphorylated and inhibited by Akt. As loss-of-function mutations of *PTEN*, which lead to persistent activation of Akt, are often found in human prostate cancer cells (e.g., PC3 and LNCaP cells) [25], the recent observation that AMPK phosphorylates and activates TSC2 suggests yet another mechanism for the inhibitory effect of AMPK on their growth [19].

Although AMPK activation by AICAR, metformin or TZDs, or expression of constitutively active mutants of AMPK has been shown to cause the death or attenuate the growth of some cancer cells (e.g., the prostate cancer cells used here) [35–37], it can inhibit the death of other cells. The latter include astrocytes and endothelial cells exposed to the fatty acid palmitate [38–40] and some cancer cells when deprived of glucose [41,42]. Although the reason for these apparently opposing effects of AMPK activation is not known, it is noteworthy that the protective effect of AMPK has been observed principally in cells that are not dividing (e.g., confluent cells) and whose survival is threatened by acute stresses. As noted earlier, in such cells, the predominant effect of AMPK appears to be to stimulate processes that generate ATP and inhibit others that consume ATP and are not acutely necessary for survival. Possibly in dividing cells, the inhibition of ATP consuming processes by AMPK may be less compatible with their survival. In keeping with this notion, Cantley and co-workers [40] have proposed a model in which LKB1, by influencing AMPK activity, has a dual function. They suggested that LKB1 functions as a tumor suppressor in cells with a normal energy status by virtue of AMPK inhibition of mTOR and other anabolic processes, whereas its principal action in cells (including certain tumor cells) in which the AMP/ATP ratio is increased by stresses such as glucose deprivation and hydrogen peroxide is anti-apoptotic. We would suggest based on our preliminary data that the tumor suppressor effect dominates in cultured prostate cancer cells.

In conclusion, AMPK is activated in prostate cancer cells by both AICAR and the TZD rosiglitazone. When this occurs, de novo fatty acid synthesis and the enzymes that regulate it are inhibited, as are mTOR and p70S6K. In addition, the expression of the cell cycle inhibitor p21 is increased. Whether this triple effect makes prostate cancer cells especially vulnerable to the growth-inhibitory action of AMPK activation remains to be determined.

Acknowledgments

This work was in part supported by National Institutes of Health Grants GM05959 (to Z. Luo) and DK19514 (N. Ruderman).

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