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The Interplay of AMP-activated Protein Kinase and Androgen Receptor in Prostate Cancer Cells†

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Abstract

AMP-activated protein kinase (AMPK) has recently emerged as a potential target for cancer therapy due to the observation that activation of AMPK inhibits tumor cell growth. It is well-known that androgen receptor (AR) signaling is a major driver for the development and progression of prostate cancer and that downregulation of AR is a critical step in the induction of apoptosis in prostate cancer cells. However, little is known about the potential interaction between AMPK and AR signaling pathways. In the current study, we showed that activation of AMPK by metformin caused decrease of AR protein level through suppression of AR mRNA expression and promotion of AR protein degradation, demonstrating that AMPK activation is upstream of AR downregulation. We also showed that inhibition of AR function by an anti-androgen or its siRNA enhanced AMPK activation and growth inhibition whereas overexpression of AR delayed AMPK activation and increased prostate cancer cellular resistance to metformin treatment, suggesting that AR suppresses AMPK signaling-mediated growth inhibition in a feedback mechanism. Our findings thus reveal a novel AMPK-AR regulatory loop in prostate cancer cells and should have a potential clinical significance.

Keywords

AMP-activated protein kinase; Androgen receptor; Prostate cancer; Metformin; Apoptosis

Introduction

Prostate cancer is the most commonly diagnosed cancer and the second leading cause of cancer-related death in men in the United States (Siegel et al., 2012). It is well-known that androgen receptor (AR) signaling plays a critical role in the development and progression of

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prostate cancer (Richter et al., 2007). Androgen deprivation therapy (ADT) has been the mainstay of treatment for advanced/metastatic prostate cancer for a long time. Despite the initial response to ADT, almost all patients eventually progress to a stage of castration-resistant prostate cancer (Feldman and Feldman, 2001). It is worth noting that, in most cases, the disease progression at castration-resistant stage is still dependent on AR signaling pathway (Chen et al., 2004). Therefore, AR is a druggable target for all stages of the disease.

AMP-activated protein kinase (AMPK) has recently drawn more and more attention for being a potential target in cancer therapy. The idea was initially derived from the fact that AMPK is a major mediator of the function of metformin, a widely prescribed anti-diabetic drug, and that consumption of metformin, a pharmacological activator of AMPK, is associated with reduced cancer risk (Evans et al., 2005; Zhou et al., 2001). AMPK is a highly conserved serine/threonine kinase that serves as a metabolic sensor for the maintenance of cellular energy homeostasis. It is a heterotrimer consisting of one catalytic subunit (α) and two regulatory subunits (β and γ) (Woods et al., 1996). Phosphorylation of AMPK α at Thr172 is required for its catalytic activity (Hawley et al., 1996; Stein et al., 2000). AMPK β is the scaffold that connects AMPK α and AMPK γ (Woods et al., 1996). AMPK γ senses intracellular AMP:ATP ratio and facilitates AMPK α activation upon AMP binding (Cheung et al., 2000). Several upstream serine/threonine kinases have been identified as capable of phosphorylating AMPK α , including liver kinase B1 (LKB1, also known as STK11) (Hawley et al., 2003), calcium/calmodulin-dependent protein kinase kinase β (CaMKK β) (Hawley et al., 2005), and transforming growth factor β -activated kinase 1 (TAK1, also known as MAP3K7) (Momcilovic et al., 2006). Activated AMPK in turn phosphorylates its substrates such as acetyl-Coenzyme A carboxylase (ACC) at Ser79 (Davies et al., 1990), regulatory-associated protein of mTOR (Raptor) at Ser792 (Gwinn et al., 2008), and tuberous sclerosis protein 2 (TSC2) at Ser1345 (Inoki et al., 2003).

AMPK is activated under conditions that increase AMP:ATP ratio such as nutrition deprivation, hypoxia, ischemia and heat shock. Besides metformin, AMPK can be activated by several other pharmacological activators such as resveratrol (Hwang et al., 2007), berberine (Turner et al., 2008), 5-aminoimidazole-4-carboxamide riboside (AICAR) (Corton et al., 1995), A-769662 (Cool et al., 2006), PT1 (Pang et al., 2008) and salicylate (Hawley et al., 2012) through different mechanisms of action. We have also reported that green tea polyphenol epigallocatechin gallate (EGCG) analogs and a formulated 3,3'-Diindolylmethane (B-DIM) can act as AMPK activators (Chen et al., 2012a; Chen et al., 2012b). The overall goal of AMPK activation is to restore cellular energy balance by promoting ATP generating processes meanwhile suppressing ATP consuming processes. In actively proliferating cells, it has been reported that AMPK activation caused cell cycle arrest *via* up-regulation of the p53-p21 axis (Motoshima et al., 2006).

So far, the effect of metformin on prostate cancer has been investigated in preclinical studies as well as in clinical trials. However, the potential crosstalk between AMPK and AR signaling pathways remains unknown. In the current study, we investigated the interaction between AMPK and AR in prostate cancer cell models. We found that activation of AMPK by pharmacological activator metformin reduced AR protein level through suppression of AR mRNA expression and promotion of AR protein degradation. We also demonstrated that AR is an endogenous inhibitor of AMPK signaling-mediated growth suppression and cell death induction in prostate cancer cells. Our results suggest that combination of AR inhibition therapy with metformin or other AMPK activators may benefit the therapeutic outcome of AR-positive prostate cancer.

Materials and Methods

Materials

Metformin and bicalutamide (Casodex[®]) were purchased from Toronto Research Chemicals (North York, Ontario, Canada). 6-[4-(2-Piperidin-1-ylethoxy)phenyl]-3-pyridin-4-ylpyrazolo[1,5-a]pyrimidine (Compound C) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich (St. Louis, MO). Antibodies against poly(ADP-ribose) polymerase (PARP)-1 (F-2), AR (N-20) and actin (C-11) were from Santa Cruz Biotechnology (Santa Cruz). Antibodies against AMPK α (23A3), phospho-AMPK α (Thr172) (40H9), phospho-ACC (Ser79), and phospho-Raptor (Ser792) were purchased from Cell Signaling Technology (Danvers, MA). RPMI1640, penicillin and streptomycin were obtained from Invitrogen (Carlsbad, CA) and fetal bovine serum (FBS) was from Aleken Biologicals (Nash, TX).

Cell culture

LNCaP and PC3 cells were obtained from American Type Culture Collection (Manasssa, VA). C4-2B cells were obtained from Prof. Leland Chung (Emory University, Atlanta, GA; and currently at Cedars-Sinai, Los Angeles, CA). PC3 cells overexpressing wild type AR (PC3-AR) were obtained from Dr. Fazlul Sarkar (Wayne State University, Detroit, MI). These cell lines were grown in RIMP1640 medium supplemented with 10% FBS, 100 units/ml of penicillin and 100 μ g/ml of streptomycin, and maintained in a humidified incubator at 37°C and 5% CO₂. All experiments were performed in RPMI1640 medium containing 10% regular FBS, without adding any additional AR agonist.

MTT assay

Cells were seeded in a 96-well plate at ~70% (for 24 or 48 h treatment) or ~30% confluency (for more than 48 h treatment) 24 h ahead, followed by addition of drugs as indicated. After drug incubation, the media was removed and 100 μ l of MTT (1 mg/ml) was added. After 2 h incubation at 37°C, MTT was removed and 100 μ l of DMSO was added to dissolve the purple formazon crystals. Colorimetric analysis was then performed at 560 nm by Wallac Victor 3 Multilabel Counter (PerkinElmer, Boston, MA). The relative absorbance values are expressed as percentage of control (100%) and shown as means $\pm$ SD of triplicates.

DNA and siRNA transfection

For DNA transfection, PC3 cells were seeded in 60 mm dishes overnight and then transfected with AR DNA constructs (0.5 μ g/ml in the medium) using Lipofectamine LTX (Invitrogen, Carlsbad, CA) for 24 hours. Empty vector transfection served as negative control. For siRNA transfection, LNCaP cells were seeded in six-well plates overnight and then transfected with AR siRNA duplexes (2.5 μ g/ml in the medium) using RNAiFect (QIAGEN, Valencia, CA) for 72 hours. Both AR-specific siRNA (sense: 5'-GGAACUCGAUCGUAUCAUUTT-3'; antisense: 5'-AAUGAUACGAUCGAGUUCCTT-3') and negative control siRNA were ordered from QIAGEN (Valencia, CA).

Whole cell extract preparation

Whole cell extract was prepared using lysis buffer (50 mM Tris-HCl at pH 8.0, 150 mM NaCl, 0.5% NP-40) as described previously (Kuhn et al., 2003). The protein concentration was determined by Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA).

Caspase-3 activity assay

Fresh-made whole cell extract (20 μ g per sample) was incubated with 20 μ M fluorogenic caspase-3 substrate Ac-DEVD-AMC (Calbiochem, La Jolla, CA) in 100 μ L of Tris-HCl (20 mM, pH 7.5). After 2 hours incubation at 37°C, the AMC liberated from the fluorogenic substrate was detected spectrofluorometrically (λ_{ex} = 355 nm and λ_{em} = 460 nm) by Wallac Victor 3 Multilabel Counter (PerkinElmer, Boston, MA). The data are expressed as percentage of control (100%) and shown as means $\pm$ SD of triplicates.

Western blot analysis

Western blot analysis was performed using 10% or 6% SDS-PAGE and semi-dry transfer system as described previously (Kuhn et al., 2003). Densitometry was quantified using AlphaEase FC software (Alpha Innotech, San Leandro, CA).

RNA isolation, reverse transcription, and quantitative real-time PCR—Total RNA from cells was isolated using the RNeasy Mini Kit (QIAGEN, Georgetown, MD) according to the manufacturer's protocol. RNA concentration was determined by NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE). Total RNA (170 ng) was reverse transcribed with random hexamer primers using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA) according to the vendor's protocol. Two μ L of the cDNA product was subjected to quantitative real-time PCR using the StepOnePlus Real-Time PCR System (Applied Biosystems, Invitrogen) and TaqMan Fast Universal PCR Master Mix (Applied Biosystems, Invitrogen). All primers and TaqMan probes were purchased from the Applied Biosystems inventory (Invitrogen). All samples were measured in triplicates and normalized to the values for GAPDH. Data are expressed as fold change of 0 hour control (1.00) and shown as mean $\pm$ SD of triplicates.

Results

Decreased AR protein levels following AMPK activation in AR-positive prostate cancer cells

Toward the goal of understanding the physiological roles of AR and AMPK in prostate cancer cell growth and death, we first measured the effect of metformin in AR-positive prostate cancer LNCaP and C4-2B cells. Both cell lines were treated with different concentrations of metformin for 48 hours followed by measurement of growth inhibition using MTT assay. Both LNCaP and C4-2B cell lines showed a dose-dependent growth inhibition after metformin treatment (Fig. 1A). To explore the molecular mechanism responsible for metformin-mediated growth inhibition, we performed a kinetic experiment in which LNCaP or C4-2B cells were treated with 30 mM metformin for 2, 4, 8, 12, 16 or 24 hours. We observed that AMPK activation in both cell lines occurred as early as 2 hours after treatment, manifested by increased levels of phospho-AMPK α (Thr172) as well as its downstream target phospho-ACC (Ser79), whereas the total AMPK α level remained the same (Fig. 1B, left and right panels). Importantly, following AMPK activation, AR protein level decreased significantly in a time-dependent manner (Fig. 1B). Following AR protein level decrease, a significant induction of apoptosis, as measured by increased levels of caspase-3 activity and PARP cleavage, was observed after 24-hour metformin treatment (Fig. 1B–C). These results suggest that metformin-induced AMPK activation leads to a decrease in AR protein expression level which contributes to apoptosis induction in AR-positive prostate cancer cells.

To elucidate the mechanism(s) through which AMPK activation decreases AR protein level, we performed a quantitative real-time PCR analysis to measure the change of AR mRNA expression over the same time period of metformin treatment. As shown in Figure 1D, the

expression of AR mRNA decreased in a time-dependent manner (black solid line), which matched the decrease of AR protein level (grey dash line, quantification of AR protein from Fig. 1B, left panel), suggesting that the decrease of AR protein level is, at least partially, due to the decrease of AR mRNA expression. These data also indicate that AR protein decrease is a direct effect of AMPK activation rather than a consequence of apoptosis.

To test if chronic exposure to a lower dose metformin can also induce AR protein decrease and cell death, we treated LNCaP and C4-2B cells with 1.25, 2.5, 5 or 10 mM metformin daily for up to 5 days. In both cell lines, we observed a dose- and time-dependent cell growth inhibition in response to metformin treatment (Fig. 2A, B). We further studied protein expression and phosphorylation profile in LNCaP cells with 5 mM metformin daily treatment for up to 5 days. The phospho-AMPK α level increased gradually, along with the increase of phospho-ACC level, whereas the total AMPK α level remained the same throughout the experiment (Fig. 2C). Accompanied with AMPK activation was the gradual decrease of AR protein level and the full length of PARP, an apoptotic indicator (Fig. 2C). These results verified that metformin in low millimolar range was also capable of inducing AR protein decrease and inhibiting prostate cancer cell growth. Taken together, the above data reveals AR protein level decrease as one of the downstream events of AMPK activation.

Inhibition of AR function facilitates metformin-induced AMPK activation and growth inhibition in AR-positive prostate cancer cells

That AR is located downstream of AMPK activation (Figs. 1, 2) suggests that AR might function as an endogenous inhibitor of AMPK-mediated prostate cancer cell growth suppression. If so, we would predict that targeting and inhibiting AR could facilitate AMPK signaling. For this purpose, we used bicalutamide, a clinically used non-steroidal anti-androgen for the treatment of prostate cancer (Kolvenbag et al., 1998). We first compared the growth inhibitory effect of metformin plus bicalutamide to that of each drug alone in AR-positive LNCaP and C4-2B cells by MTT assay. Bicalutamide augmented metformin-induced growth inhibition in both cell lines (Fig 3A, B). To understand the involved molecular mechanism, we performed a kinetic experiment using LNCaP cells treated with metformin, bicalutamide or metformin plus bicalutamide for up to 24 hours, followed by measuring AMPK signaling and AR status (Fig. 3C). Compared to metformin treatment alone, combination of bicalutamide plus metformin induced an earlier (2 h vs. 4 h) and higher (up to 5.0 fold vs. 2.3 fold at 4–8 h) level of AMPK activation, manifested by phosphorylation of AMPK α (Fig. 3C, lanes 7–12 vs. 1–6). Consistently, we observed earlier phosphorylation of AMPK downstream targets ACC (at Ser79) and Raptor (at Ser792) in the combination group (Fig. 3C). In comparison, bicalutamide itself had little effect on AMPK activation and there was little difference in total AMPK α protein level in all treatment groups (Fig. 3C). Along with prompt and enhanced AMPK activation induced by the combination treatment, earlier AR protein level decrease (8 h vs. 24 h) was also observed (Fig. 3C). These data support the conclusion that AR is an inhibitor of AMPK signaling in prostate cancer cells. Taken together, these data demonstrate that addition of bicalutamide promotes metformin-induced AMPK activation and AR protein level decrease in AR-positive prostate cancer cells.

AR inhibits AMPK activation

To further examine the hypothesis that AR is an inhibitor of AMPK signaling-mediated growth suppression in prostate cancer cells, we determined whether knockdown of AR could facilitate metformin-induced AMPK activation. In this experiment, AR-positive prostate cancer LNCaP cells were transfected with scramble or AR-specific siRNA for 72 hours, followed by treatment of metformin at 10 and 20 mM for 4 hours. Compared to control

cells, knockdown of AR resulted in significantly higher level of phospho-AMPK α induction upon metformin treatment, while total AMPK α protein remained unchanged (Fig. 4A), confirming that AR plays an inhibitory role upstream of AMPK activation, but not on AMPK protein level.

If AR is an inhibitor of AMPK signaling-mediated growth inhibition in prostate cancer cells, reintroduction of AR into AR-negative cells should be able to generate resistance to metformin. To test this idea, we compared the efficacy of metformin in parental PC3 cells (AR-negative) and PC3 cells with stable transfection of AR (PC3-AR) by MTT assay. As shown in Fig. 4B, PC3-AR cells exhibited much higher resistance to metformin than parental PC3 cells, associated with reduction of transfected AR protein expression at late time points (data not shown).

We then studied the effect of AR on metformin-induced AMPK activation in PC3 cells transiently transfected with AR construct. Compared to empty vector control, re-expression of AR in PC3 cells delayed metformin-induced AMPK activation from 4 h to 24 h whereas the total AMPK α levels were unchanged (Fig. 4C, lanes 4–6 vs. 1–3). Taken together, these data suggest that reintroduction of AR into AR-negative prostate cancer cells generates resistance to metformin by suppressing AMPK activation, further confirming that AR is an inhibitor of AMPK signaling-mediated growth suppression in prostate cancer cells.

Inhibition of AMPK signaling prevented metformin-induced AR degradation

Finally, we studied whether metformin-induced AR protein downregulation could be blocked by compound C, a chemical AMPK inhibitor (Zhou et al., 2001). Exponentially growing LNCaP cells were pretreated with 20 μ M of compound C or the solvent DMSO for 12 hours, followed by metformin co-treatment for up to 30 hours. Cells treated with only compound C were also included as control. In untreated control cells, AR antibody recognized several bands with molecular weight lower than full-length AR (110 kDa), which could be the basal level of AR degradation fragments (Fig. 5, lanes 1 and 13). These bands increased in cells treated with metformin, suggesting that, in addition to suppressed expression of AR mRNA (Fig. 1D), metformin treatment also causes AR protein degradation (Fig. 5, lanes 2–5 vs. 1). Importantly, compared to the DMSO-pretreated cells, pretreatment with compound C prevented the generation of AR degradation fragments induced by metformin (Fig. 5, lane 7–12 vs. lane 1–6). As expected, compound C also suppressed AMPK signaling as evident by decreased levels of its downstream substrates, phospho-Acc and phospho-Raptor (Fig. 5, lanes 7–12 vs. 1–6). No AR degradation fragments were observed in cells treated with only compound C, either (Fig. 5, lanes 14–19). These results verify that activation of AMPK signaling is required for both induced and basal levels of AR protein degradation.

Discussion

In the current study, we showed that (i) activation of AMPK by metformin caused decrease of AR protein level through suppressed expression of AR mRNA and promoted degradation of AR protein; and (ii) AR inhibited AMPK signaling-mediated growth suppression and apoptosis in prostate cancer cells by suppressing AMPK activation. Our findings thus reveal a novel AMPK-AR regulatory loop that involves in regulating prostate cancer cell growth and death (Fig. 6).

AR signaling is crucial in prostate cancer development and progression by regulating cell proliferation, differentiation and apoptosis. Downregulation of AR is a critical step in the induction of apoptosis in prostate cancer cells under various cytotoxic stimuli. For example, a study by Li *et al* demonstrated that AR degradation is a direct effect of berberine, rather

than a bystander effect of apoptosis (Li et al., 2011). Similarly, a novel curcumin analogue, B-DIM, was found to enhance androgen receptor degradation activity, thereby leading to inhibition of proliferation and induction of apoptosis in prostate cancer cells (Shi et al., 2009). In the current study, we showed that metformin-induced AMPK activation caused AR protein decrease by suppression of AR mRNA expression and promotion of AR protein degradation (Figs. 1, 2 & 5). Additionally, metformin-induced AR protein decrease was partially prevented by the presence of dihydrotestosterone (DHT), an AR agonist (data not shown). These data clearly suggest that the decrease of AR protein is a direct effect of AMPK activation rather than a consequence of apoptosis. In our experimental settings, this was no obvious evidence suggesting that AMPK activation directly suppressed AR function. Therefore, metformin-induced AMPK activation mainly suppressed AR protein level and consequently inhibited AR functions. This finding sheds light on a new pathway that can lead to downregulation of AR protein. It has been reported that AR could be degraded by different proteases including the ubiquitin-proteasome system (Sheflin et al., 2000), caspase-3 (Lin et al., 2004), and calpain (Pelley et al., 2006; Yang et al., 2008). However, under our experimental condition, we found that proteasome inhibitor bortezomib could not prevent AR protein decrease induced by metformin (data not shown), suggesting the involvement of a non-proteasomal proteolytic activity in AMPK activation-induced AR degradation.

In the current study, we also showed that AR negatively regulates AMPK signaling-mediated growth inhibition in prostate cancer cells by suppressing AMPK activation (Figs. 3, 4). Besides phosphatases such as protein phosphatase 2C (PP2C) which directly dephosphorylates AMPK α at Thr172 (Moore et al., 1991), a couple of other proteins have been reported to negatively regulate AMPK activity through different mechanisms. For example, Akt has been found to phosphorylate AMPK α at Ser485/491 which reduces phosphorylation of AMPK α at Thr172 that is required for AMPK activity (Horman et al., 2006). Similarly, Djouder *et al* reported that protein kinase A (PKA) directly associates with and phosphorylates AMPK α 1 at Ser173 to impede Thr172 phosphorylation by LKB1 (Djouder et al., 2010). More recently, it was reported that the orphan nuclear receptor Nur77 negatively regulates AMPK activation by binding to and sequestering LKB1 in the nucleus (Zhan et al., 2012). Nowadays, the nonclassical effects of nuclear receptor have drawn more and more attention (Wehling and Losel, 2006). For example, in addition to classical intracellular AR, the membrane-bound form of AR has been identified and found to mediate the fast, nongenomic effects of steroid hormones (Kampa et al., 2002). Furthermore, the identification of numerous transcript variants of AR in various prostate cell lines and clinical samples, most of which lack partial or entire DNA binding domain, also stimulated the study on hormone-independent effects of AR (Gonit et al., 2011; Haile and Sadar, 2011). The androgenic effects of AR in prostate cancer are principally genomic. However, less is known about the molecular mechanisms by which AR serves the anabolic effects of testosterone. They could be both genomic and non-genomic. Given the anabolic effects of metformin in muscle, it is unlikely that the AMPK-AR regulatory mechanism described in our study here contributes to the anabolic effects of androgens in muscle, which however needs to be determined. Further investigation on how AR suppresses AMPK activation in prostate cancer cells is needed to unravel the underneath mechanism.

With respect to the concern of the apparent discrepancy between the concentrations of metformin used in cultured cells *vs.* in patients, there are several explanations. Typically the concentration of metformin used to activate AMPK in cultured cells is 1–20 mM. These are 1–2 orders of magnitude higher than the concentrations estimated to occur in human plasma (10–40 μ M) following a therapeutic dose of around 30 mg/kg. One explanation is that many cultured cell lines lack the expression of OCT1 transporter that is required to transport biguanide compounds like metformin into the cell (Fogarty and Hardie, 2010). Secondly,

data from *in vivo* study showed that metformin accumulates in multiple tissues of diabetic mice in concentrations several fold higher than those in blood, with the greatest accumulation occurred in the small intestine (Wilcock and Bailey, 1994). These observations suggest that it may be possible to reach therapeutic levels in tumor tissue when used for cancer treatment. A third explanation for the discrepant concentration of metformin used in cultured cells and in patients involves the non-physiological growth conditions used in the cell culture models to assess the *in vitro* growth inhibitory effect of metformin. The majority of cancer cell lines are maintained in non-physiological conditions that are optimized for maximum growth and proliferation with extremely high amounts of growth factors and glucose in culture media, which may account for the elevated doses of metformin required to elicit cellular responses *in vitro* (Dowling et al., 2012).

Although most studies showed that AMPK activation induced growth arrest and/or cell death in a variety of cancer cell types, a few studies have conversely reported that AMPK could serve as a tumor promoter in prostate cancer and astrocytoma under certain conditions. For example, CaMKK β , one of the AMPK upstream kinases, was found to be overexpressed in both hormone-sensitive and -resistant prostate cancer cells, and CaMKK β -mediated activation of AMPK was found to be required for androgen-dependent migration of prostate cancer cells (Frigo et al., 2011; Massie et al., 2011). In the case of astrocytoma, Rios *et al* reported that oncogenic HRas mutation and Pten deletion leads to AMPK activation, and this activation is required to maintain cancer cell proliferation, which may involve AMPK-mediated phosphorylation of Rb protein (Rios et al., 2013). These findings stressed the necessity of proper interpretation of AMPK signaling according to different contexts. In addition, the isoform composition and the subcellular localization of AMPK complex might also affect the consequences of AMPK activation.

In summary, our current study demonstrated that while AMPK activation leads to decrease of AR protein expression, AR in turn suppresses AMPK signaling-mediated growth inhibition in prostate cancer cells. These findings have established a new connection between AMPK and AR signaling pathways, and provided preclinical evidence that administration of metformin or other AMPK activators may benefit the therapeutic outcome of AR-positive prostate cancer.

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Figure 1. Metformin induces AMPK activation and subsequent decrease in AR mRNA and protein levels in AR-positive prostate cancer cells
 (A) LNCaP and C4-2B cells were treated with metformin at concentrations of 5, 10, 20 or 30 mM for 48 hours, followed by MTT assay. (B–C) LNCaP and C4-2B cells were treated with 30 mM metformin for up to 24 hours, followed by Western blot analysis (B) and caspase-3 activity assay (C). p-AMPK α , AMPK α phosphorylated at Thr172; p-ACC, ACC phosphorylated at Ser79. (D) LNCaP cells were treated with 30 mM metformin for up to 24 hours, followed by quantitative real-time PCR analysis. Black solid line is the quantification of AR mRNA expression after normalizing to GAPDH; data are expressed as fold change of 0 hour control (1.00) and shown as mean $\pm$ SD of triplicates. Grey dash line is the quantification of AR protein expression in the left panel of Fig. 1B after normalizing to actin.

Figure 2. Metformin in low millimolar range induces AMPK activation, AR protein decrease and apoptosis in prostate cancer cells
 (A, B) LNCaP (A) and C4-2B (B) cells were treated daily with metformin at concentrations of 1.25, 2.5, 5 or 10 mM for up to 5 days, followed by MTT assay. (C) LNCaP cells were treated with 5 mM metformin for 24, 48, 72, 96 or 120 hours, followed by Western blot analysis.

Figure 3. Bicalutamide promotes metformin-induced AMPK activation, AR degradation and growth inhibition in AR-positive prostate cancer cells

(A, B) LNCaP (A) and C4-2B (B) cells were treated with various concentrations of metformin in the presence or absence of bicalutamide (Bic) at indicated concentrations for 24 hours, followed by MTT assay. (C) LNCaP cells were treated with metformin (30 mM) alone or in combination with bicalutamide (20 μ M) for 0, 2, 4, 8, 12 or 24 hours, followed by Western blot analysis. p-Raptor, Raptor phosphorylated at Ser792.

Figure 4. AR negatively regulates metformin-induced AMPK activation in prostate cancer cells (A) AR-positive LNCaP cells were transfected with AR-specific siRNA (lanes 4–6) or scramble control (lanes 1–3) for 72 hours, and then treated with 10 or 20 mM metformin for 4 hours, followed by Western blot analysis. (B) AR-negative parental PC3 cells and AR-transfected PC3-AR cells (stable transfection) were treated with metformin at 5, 10, 20 or 30 mM for 48 hours, followed by MTT assay. (C) PC3 cells were transiently transfected with AR construct (lanes 4–6) or empty vector (lanes 1–3) for 24 hours, and then treated with 30 mM metformin for 4 or 24 hours, followed by Western blot analysis.

Figure 5. Compound C prevents metformin-induced AMPK activation and AR degradation in prostate cancer cells

LNCaP cells were pretreated with either 0.1% DMSO (lanes 1–6) or 20 μ M compound C (CC) (lanes 7–12) for 12 hours and then co-treated with 30 mM metformin (Met) for additional 0, 4, 8, 16, 24 or 30 hours (lanes 1–12), or treated with 20 μ M CC for 0, 12, 16, 20, 28, 36 or 42 hours (lanes 13–19). Cell lysates were prepared and subjected to Western blot analysis.

Figure 6. Schematic diagram represents the regulatory loop of AR and AMPK in prostate cancer cells

See Discussion for details.