

PI3K-Akt signaling is involved in the regulation of p21^{WAF/CIP} expression and androgen-independent growth in prostate cancer cells

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Abstract. The purpose of this study is to investigate the role of PI3K-Akt signaling in prostate cancer cell growth and androgen receptor (AR)-mediated gene expression. Androgen-dependent LNCaP cells and their androgen-independent counterpart, LNCaP-AI cells, were used. We found that PI3K-Akt signaling is elevated in LNCaP-AI cells compared to that in LNCaP cells and is involved in androgen-independent growth. More importantly, PI3K-Akt signaling enhances AR activity and is involved in the induction of AR target genes, such as p21^{WAF/CIP}, a gene with anti-apoptosis activity and associated with androgen-independent growth in human prostate cancer. A receptor tyrosine kinase inhibitor also inhibits the PI3K-Akt signaling and compromises AR activity and cell growth. These findings suggest that the PI3K-Akt cell growth survival pathway and its downstream-regulated gene, p21^{WAF/CIP}, are targets for developing novel therapies against prostate cancer, especially those androgen-independent diseases.

Introduction

Hormone ablation therapy is a mainstay treatment for advanced prostate cancer (stage D, local and distant metastasis) (1-3). Most prostate cancers, however, become androgen-independent (hormone-refractory) within 6 to 18 months (4,5). It is not clear how prostate cancer cells make the transition from being androgen-dependent to being androgen-independent after hormone ablation therapy.

AR is expressed in most hormone refractory prostate cancers and elevated AR activity is involved in prostate cancer

progression (6,7). The key question is, how is AR activated under low androgen (hormone ablation) conditions? AR mutation and gene amplification are common alterations associated with progression (8-11). Mutations in the ligand binding domain generate promiscuous AR, which accepts a broad ligand spectrum for activation, such as by unfavorable adrenal androgen and androgen antagonists (12). In addition, overexpression of steroid hormone receptor coactivators, such as SRC1, TIF-2, and ARA55, dramatically enhances AR activity (13,14). More importantly, AR can be activated by phosphorylation (15,16). For instance, HER-2/neu (erbB2), neuroendocrine, and phosphoinositide 3-kinase (PI3K)-Akt-mediated signal transduction pathways have been demonstrated to enhance AR and are associated with prostate cancer development and progression (17-21).

The PI3K-Akt pathway has been described as a dominant growth survival pathway in prostate cancer. Elevated PI3K-Akt signaling is correlated with prostate cancer progression (19,22-24). The PI3K-Akt pathway transduces signals from multiple growth factors and cytokines, including epidermal growth factor (EGF), transforming growth factor (TGF), nerve growth factor (NGF), neuropeptides, and Interleukin-6 (IL-6) and regulates cell proliferation, survival, and motility, which are critical for tumorigenesis (25-27). Upon binding by growth factors, their tyrosine kinase receptors or G-protein-coupled receptors recruit PI3K to the inner surface of the plasma membrane and activate PI3K. Phosphatidylinositol 3,4,5 triphosphate (PI3,4,5P or PIP3), the lipid product of PI3K, triggers phosphorylation and activation of the downstream serine/threonine kinase Akt, which promotes cell survival by modulating key proteins with various biological functions, such as phosphorylation and inactivation of the pro-apoptotic molecules, Bad and Caspase 9 (23,24). On the other hand, the lipid phosphatase, PTEN, a tumor suppressor, dephosphorylates PIP3 back to PIP2 and thus shuts off PI3K-Akt signaling.

The cyclin-dependent kinase (CDK) inhibitor, p21^{WAF/CIP}, is a multifunctional protein involved in cell-cycle control, DNA repair, and anti-apoptosis (28,29). p21^{WAF/CIP} was first identified as a cell-cycle inhibitor. It binds to and inhibits CDKs, blocking the cell cycle in G1/S or G2/M phase. Subsequently, p21^{WAF/CIP} was found to have anti-apoptotic activity in various systems (30,31). For instance, p21^{WAF/CIP} protects cells from apoptosis induced by p53 or γ -radiation by binding to and inhibiting

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c-Jun N-terminal kinase 1 (JNK1, also named stress-activated protein kinase, SAPK) (32,33). p21^{WAF/CIP} interacts with JNK1 via its N-terminal domain, the same domain that interacts with CDKs. More importantly, recent findings suggested that p21^{WAF/CIP} overexpression provides cancer cells with a survival advantage and is a target for cancer therapy (34).

In prostate cancer, p21^{WAF/CIP} is expressed at a low level in the normal prostatic epithelium (35,36) and heterogeneously overexpressed in a certain percentage of prostate cancer cells. p21^{WAF/CIP} is more commonly overexpressed in poorly differentiated and rapidly proliferating tumors and is often co-expressed with Bcl-2 (37-39). Upon hormone ablation therapy, the rate of p21^{WAF/CIP}-positive cases significantly increased, independent of p53, an upstream regulator. p21^{WAF/CIP} overexpression is associated with prostate cancer progression and its androgen-independent growth (40-43).

In the current study, we determined the molecular mechanisms involved in androgen-independent growth and AR target gene expression in prostate cancer cells.

Materials and methods

Cell culture. The androgen-dependent human prostate adenocarcinoma cell line, LNCaP (also named LNCaP-FGC, ATCC, Rockville, MD), was maintained in RPMI-1640 (Invitrogen, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS, HyClone Laboratories, Logan, UT) (complete medium) at 37°C in 5% CO₂. The androgen-independent LNCaP-AI cells were currently maintained in RPMI-1640 supplemented with 10% charcoal/dextran-treated FBS (HyClone Laboratories, Logan, UT) (stripped medium) (44).

Reagents. LY294002, Wortmannin, and AG879 were purchased from CalBiochem (EMD Biosciences Inc., La Jolla, CA). 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), dehydrotestosterone (DHT), and anti-β-actin antibody were obtained from Sigma Chemicals (St. Louis, MO). Antibodies against Akt and phospho-Akt were purchased from Cell Signaling Technology Inc. (Beverly, MA). Anti-PSA antibody was from Dako Corp. (Carpinteria, CA).

Plasmids. PSA(-4.3)-Luc and pGL3-ARE-E4-Luc are gifts from Zhengxin Wang, Department of Cancer Biology, M.D. Anderson Cancer Center, Houston, TX. pUS-Akt-act containing a constitutively active Akt, pUS-Akt-DN containing a dominant negative Akt, and empty vector pUS-amp were purchased from Upstate Biotechnology (Charlottesville, VA).

MTT assay. Tumor cell growth was estimated using MTT assay as previously described (45). Briefly, LNCaP and LNCaP-AI cells were seeded into 96-well cell culture plates at a density of 5x10³ cells/well in stripped medium. After incubation in 5% CO₂ at 37°C overnight, LNCaP cells were treated with various concentrations of drugs in stripped medium supplemented with 10⁻⁸ M DHT and LNCaP-AI cells were treated in stripped medium for 5 days. At the end of incubation, 20 μl of MTT (2.5 mg/ml in phosphate-buffered saline, PBS) was added to each well, and the cells were further incubated for 1 h at 37°C to allow a complete reaction between the dye and the enzyme mitochondrial dehydrogenase in the viable

cells. After removal of the residual dye and medium, 100 μl of dimethylsulfoxide was added to each well, and the absorbance at 570 nm was measured using a BMG microplate reader (BMG Labtech, Inc., Durham, NC).

Western blot analysis. Western blot analysis was performed as previously described (45). Briefly, aliquots of samples with the same amounts of protein, determined using the Bradford assay (Bio-Rad, Hercules, CA), were mixed with loading buffer [final concentrations of 62.5 mM Tris-HCl (pH 6.8), 2.3% SDS, 100 mM dithiothreitol, and 0.005% bromophenol blue], boiled, fractionated in an SDS-PAGE, and transferred onto a 0.45-μm nitrocellulose membrane (Bio-Rad). The filters were blocked with 2% fat-free milk in PBS, and probed with first antibody (0.05 μg/ml IgG) in PBS containing 0.1% Tween-20 (PBST) and 1% fat-free milk. The membranes were then washed four times in PBST and incubated with horseradish peroxidase-conjugated secondary antibody in PBST containing 1% fat-free milk. After washing four times in PBST, the membranes were visualized using the ECL Western blotting detection system (Amersham Co., Arlington Height, IL).

Transient transfection assay. LNCaP or LNCaP-AI cells (2x10⁵) were seeded in 12-well tissue culture plates. On the following day, lipofectin-mediated transfection was used for the transient transfection assay according to the protocol provided by Invitrogen Inc. Then, the cells were treated with hormone or drugs in stripped medium for 24 h. Subsequently, the cell extracts were prepared according to the *in vitro* luciferase assay kit (Promega, Madison, WI). Luciferase assays were performed using the Berthold detection system (Pforzheim, Germany). For each assay, cell extract (20 μl) was used and the reaction was started by injection of 50 μl of luciferase substrate. Each reaction was measured for 10 sec in the Luminometer. Luciferase activity was defined as light units/mg protein.

Results

PI3K-Akt signaling is involved in androgen-independent growth in LNCaP-AI cells. We examined the effects of PI3K inhibitors, LY294002 and Wortmannin, and receptor tyrosine kinase inhibitor, AG879, on cell growth. LNCaP cells were cultured in stripped medium supplemented with DHT and LNCaP-AI cells in stripped medium for 5 days. As shown in Fig. 1, LY294002 and Wortmannin significantly inhibited the growth of both LNCaP and LNCaP-AI cells in a dose-dependent fashion (Fig. 1A and B). The growth of both LNCaP and LNCaP-AI cells was also inhibited in a dose-dependent manner by Tyrphostin AG879, a small molecule blocking tyrosine kinase activities of NGF and HER2/Neu receptors (Fig. 1C) (46,47). Interestingly, LNCaP-AI cells were more susceptible than LNCaP cells to the growth inhibitory effects of both the PI3K inhibitors (Fig. 1A and B) and the receptor tyrosine kinase inhibitor (Fig. 1C).

The signaling level of the PI3K-Akt pathway is elevated in LNCaP-AI cells. To determine the signaling levels of the PI3K-Akt pathway in LNCaP and LNCaP-AI cells and to

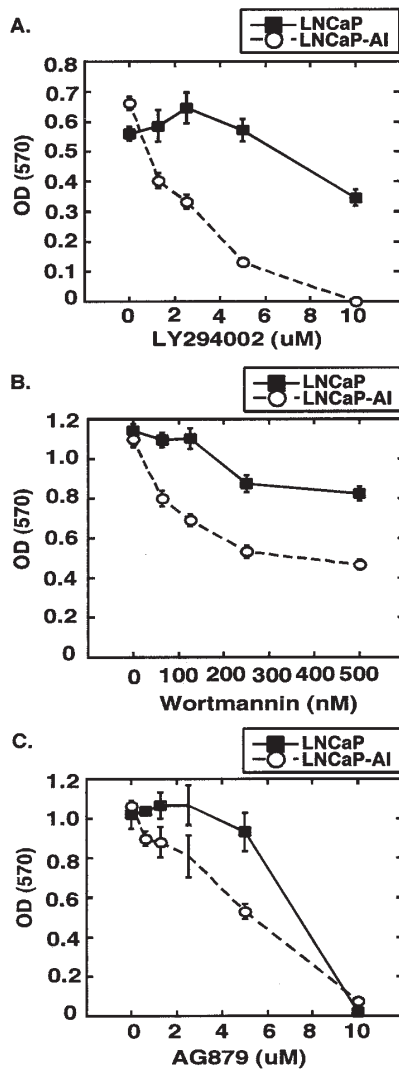


Figure 1. Cell growth experiments. LNCaP cells were cultured in stripped medium supplemented with DHT (10^{-8} M) and LNCaP-AI cells in stripped medium. The cells were treated with LY294002 (A), Wortmannin (B), or AG879 (C) at various concentrations for 5 days, followed by MTT assay. The error bar represents the average of triplicate samples of each experiment. Each MTT assay has been repeated more than three times.

verify the inhibitory effect of PI3K inhibitors on PI3K-Akt signaling, we examined the phosphorylation level of Akt, the downstream target of PI3K, by Western blot analysis using an anti-phospho-Akt(ser473) antibody. The elevated phosphorylation of Akt represents enhanced signaling of the PI3K-Akt pathway. We found that the phosphorylation level of Akt was elevated in LNCaP-AI cells relative to that in LNCaP cells, while the protein level of Akt remained similar in these two cell lines (Fig. 2A). LY294002 inhibited Akt phosphorylation in a dose-dependent manner in both LNCaP and LNCaP-AI cells. However, especially at low concentrations of LY294002, e.g. 2.5 and 5.0 μ M, there was a constitutively elevated level of Akt phosphorylation in LNCaP-AI cells. Furthermore, AG879 inhibited PI3K-Akt signaling, revealed by a reduced phosphorylation level of Akt(ser473) in both LNCaP and LNCaP-AI cells (Fig. 2B).

PI3K-Akt signaling enhances AR activity in an androgen-independent manner. We determined the role of PI3K-Akt

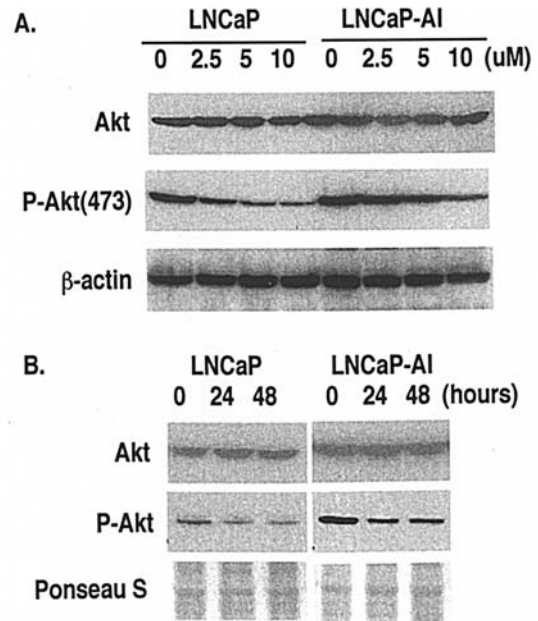


Figure 2. Phosphorylation levels of Akt upon LY294002 and AG879 treatment. LNCaP and LNCaP-AI cells were treated with LY294002 (A) for 24 h or treated with 10 μ M of AG879 (B) for 24 and 48 h, followed by Western blot analysis for Akt and phospho-Akt(473). β -actin and Ponceau S staining were served as loading control.

signaling in the regulation of PSA expression. As shown in Fig. 3A, LNCaP-AI cells expressed a reduced basal level of PSA relative to that in LNCaP cells, possibly due to long-term cell culture in stripped medium resulting in a reduced level of AR, and PSA expression was enhanced in response to androgen stimulation in both cells. LY294002 and Wortmannin inhibited the basal levels of PSA in LNCaP-AI cells, suggesting that PI3K-Akt signaling is involved in the regulation of AR activity and induction of the AR target gene (Fig. 3B and C). A robust expression of PSA was detected in LNCaP cells in the presence of DHT, which was not altered by the presence of PI3K inhibitors (Fig. 3B and C). This may be due to a relatively short term of treatment and weak effects of PI3K inhibitors as compared with a robust androgen-dependent mechanism for stimulation of a high level expression of PSA in LNCaP cells. Expression of PSA in both LNCaP and LNCaP-AI cells was inhibited by AG879 (Fig. 3D).

Next, we examined the effects of PI3K inhibitors on PSA promoter activity. As shown in Fig. 4A, Wortmannin and LY294002 significantly inhibited basal PSA promoter activity in LNCaP-AI cells in the absence of DHT. Since the PSA promoter can be regulated by multiple factors, we further determined whether this inhibition was mediated by AR. Consistently, Wortmannin and LY294002 inhibited the artificial promoter activity of the luciferase reporter, pGL3-ARE-E4-Luc, which contains four copies of androgen response elements (ARE) fused with adenovirus E4 minimal promoter at the upstream (Fig. 4B), suggesting an AR-mediated inhibition. Similarly, AG879 also significantly inhibited PSA promoter activity in LNCaP-AI cells (Fig. 4C).

To further confirm the role of PI3K-Akt signaling in regulating AR-mediated gene expression, we performed a transient transfection experiment in which LNCaP-AI cells were co-transfected with expression vector pUS-Akt-act

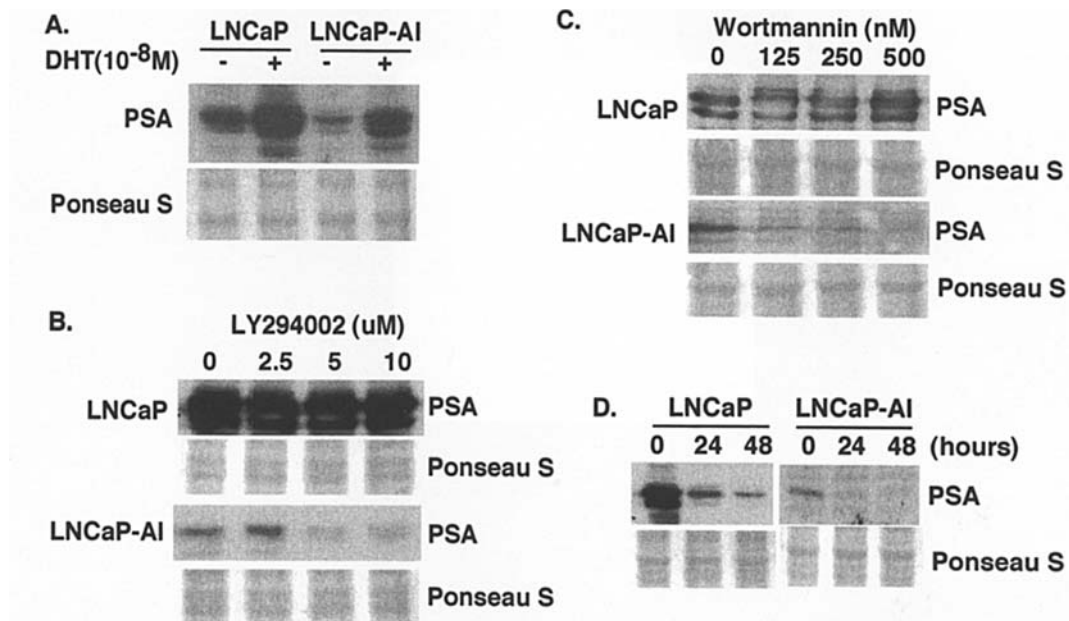


Figure 3. Western blot analysis of PSA expression. (A), LNCaP and LNCaP-AI cells were cultured in stripped medium for 5 days and then treated with DHT for 48 h. Cell extracts were prepared and analyzed using Western blotting. (B-D), LNCaP cells were cultured in stripped medium supplemented with DHT (10^{-8} M) and LNCaP-AI cells were cultured in stripped medium. The cells were treated with LY294002 (B) and Wortmannin (C) for 24 h or treated with 10μ M of AG879 (D) for 24 and 48 h, followed by cell extract preparation and Western blot analysis. Ponceau S staining as loading control.

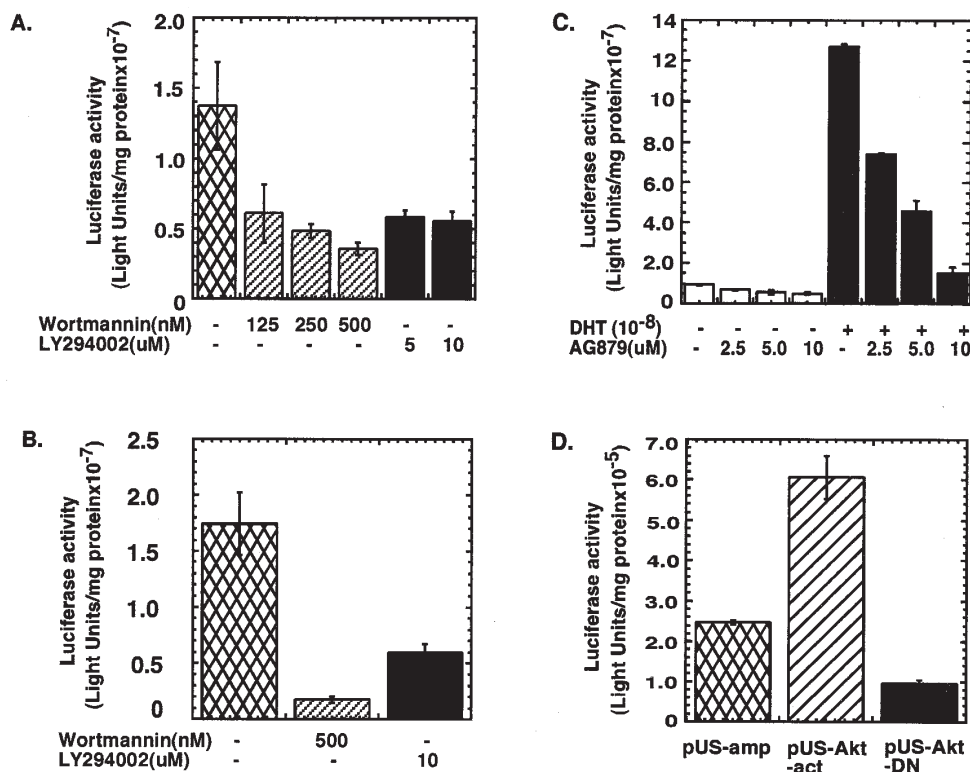


Figure 4. PI3K-Akt signaling modulates AR activity. LNCaP-AI cells were transfected with luciferase reporters PSA(-4.3)-Luc (A) and pGL3-ARE-E4-Luc (B) (0.125μ g/well in 12-well plate) and treated with Wortmannin and LY294002 in the absence of DHT for 24 h, followed by luciferase assay. (C) LNCaP-AI cells were transfected with PSA(-4.3)-Luc reporter (0.125μ g/well in 12-well plate) and treated with AG879 in the absence or presence of DHT for 24 h, followed by luciferase assay. (D) LNCaP-AI cells were cotransfected with luciferase reporter, PSA(-4.3)-Luc (0.25μ g/well in 12-well plate), with empty vector pUS-amp, pUS-Akt-act, or pUS-Akt-DN for 24 h in stripped medium, followed by luciferase assay. The error bar represents the average of duplicate samples of each experiment. Each transient transfection assay has been repeated more than three times.

containing a constitutively active form of Akt or pUS-Akt-DN containing a dominant-negative Akt. Fig. 4D shows that, in the absence of androgen, overexpression of Akt-act and Akt-DN

stimulated and inhibited PSA promoter activity, respectively. Transient overexpression of Akt-act and Akt-DN in LNCaP-AI cells similarly stimulated and inhibited the artificial ARE-

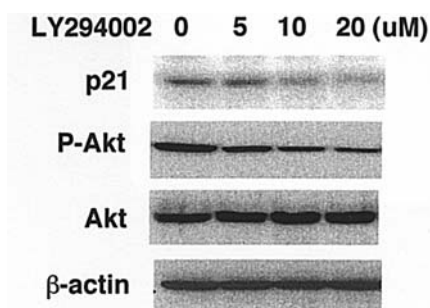


Figure 5. Regulation of p21^{WAF/CIP} expression by PI3K-Akt signaling. LNCaP cells were treated with LY294002 for 24 h. The cell extracts were prepared and subjected to Western blot analysis for p21^{WAF/CIP}, Akt, and Phospho-Akt. β-actin was served as loading control.

specific promoter from the reporter construct, pGL3-ARE-E4-Luc (data not shown). These data indicate that inhibition of the PI3K-Akt pathway compromises AR activity and AR-mediated gene expression.

These data are consistent with the effects of PI3K inhibitors on cell growth (Fig. 1) in that PI3K-Akt signaling supports both androgen-dependent and -independent growth, but preferentially androgen-independent growth, and enhances AR activity in the absence of androgen in LNCaP-AI cells.

Regulation of p21^{WAF/CIP} expression by PI3K-Akt signaling. Since the signaling level of the PI3K-Akt pathway is elevated and p21^{WAF/CIP} is also overexpressed in LNCaP-AI cells, we determined whether PI3K-Akt signaling regulates p21^{WAF/CIP} expression. We found that treatment of LNCaP cells with LY294002 significantly inhibited the endogenous p21^{WAF/CIP} expression in a dose-dependent fashion, which correlates with reduction of Akt phosphorylation (Fig. 5). Next, we investigated the role of PI3K-Akt signaling in the regulation of p21^{WAF/CIP} promoter activity. The p21(-215)-Luc reporter contains 215 base pairs of the p21^{WAF/CIP} promoter sequence, including a functional ARE (48,49). The p53 response element, located at approximately 2.4 kilobase pairs upstream of the p21^{WAF/CIP} promoter, does not include the p21(-215)-Luc reporter. Consistent with their effects on p21^{WAF/CIP} expression, PI3K inhibitors downregulated the basal activity of the p21^{WAF/CIP} promoter, while DHT stimulated the promoter activity (Fig. 6A). Furthermore, cotransfection of constitutively active Akt in LNCaP cells stimulated the p21^{WAF/CIP} promoter activity, while cotransfection of dominant negative Akt inhibited the promoter activity (Fig. 6B). A similar observation was made in LNCaP-AI cells (data not shown). These data demonstrate that PI3K-Akt signaling is involved in the induction of p21^{WAF/CIP} expression.

Discussion

The molecular mechanisms of developing androgen-independent growth in prostate cancer have not been fully appreciated. The current study determined the molecular pathway(s) involved in androgen-independent growth and androgen-regulated gene expression in our established androgen-independent prostate cancer model, LNCaP-AI cells (44). We found that Akt phosphorylation (activation)

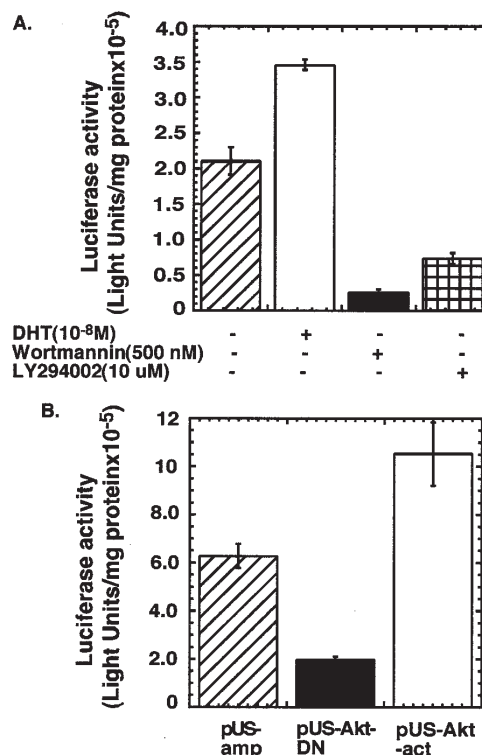


Figure 6. Regulation of p21^{WAF/CIP} promoter activity by PI3K-Akt signaling. (A), LNCaP cells were transfected with p21(-215)-Luc reporter (0.5 μg/well in 12-well plate) and treated with Wortmannin and LY294002 in the absence of DHT for 24 h, followed by luciferase assay. (B) LNCaP cells were cotransfected p21(-215)-Luc reporter with empty vector, pUS-act, pUS-Akt-act, or pUS-Akt-DN for 24 h in stripped medium, followed by luciferase assay. The error bar represents the average of duplicate samples of each experiment. These experiments have been repeated three times.

was elevated in LNCaP-AI cells compared to that in LNCaP cells. Blocking PI3K-Akt signaling by using PI3K inhibitors inhibited the growth of both LNCaP and LNCaP-AI cells in a dose-dependent manner. However, the two PI3K inhibitors produced much more significant growth inhibition in LNCaP-AI cells. The blockade of PI3K-Akt signaling by either using PI3K inhibitors or overexpression of a dominant-negative construct of Akt also reduced AR activity for stimulation of promoters of AR target genes.

Accumulating studies have demonstrated that the elevated receptor protein kinase activity by overexpression of either growth factors and cytokines or their corresponding receptors is involved in the development and progression of prostate cancer. The PI3K-Akt pathway is a major downstream signal transduction pathway in a tissue-specific or system-specific manner. We found that AG879, a receptor protein tyrosine kinase inhibitor of the NGF receptor and HER-2/neu, inhibited the PI3K-Akt signaling and androgen-dependent and -independent growth and compromised the AR activity in prostate cancer cells. In contrast with PI3K inhibitors, AG879 also inhibited PSA expression in the presence of androgen, suggesting that an additional mechanism(s) involved in AR function in a ligand-dependent manner was also inhibited by AG879.

PTEN and PI3K-Akt signaling play important roles in prostate cancer development and progression. PTEN deletion and/or mutation represent one of the most common genetic

alterations in prostate cancer and increase with disease progression (50,51). PTEN and PI3K inhibitors were shown to repress the transcriptional activity of AR, the androgen-induced cell proliferation, and the PSA production (52). Prostate-specific PTEN deletion in mice induces prostate cancer (53). PI3K-Akt signaling may directly regulate AR activity by phosphorylation and, hence, activation of AR (54) or activate AR through β -catenin, an AR coactivator (55,56). Since PTEN is mutated in LNCaP cells (57), the enhanced PI3K-Akt signaling must be due to mechanisms independent of PTEN. The mechanisms responsible for the elevated PI3K-Akt signaling in LNCaP-AI cells remain to be elucidated.

Previously, we demonstrated that p21^{WAF/CIP} is over-expressed in androgen-independent LNCaP-AI cells (44) and identified p21^{WAF/CIP} as an AR target gene (48,49). More importantly, p21^{WAF/CIP} is associated with prostate cancer progression and androgen-independent growth. In the current study, we demonstrated that the inhibition of PI3K-Akt signaling compromises p21^{WAF/CIP} expression, suggesting that this signaling pathway is involved in p21^{WAF/CIP} expression.

Unfortunately, data from different cell lines and from different labs seem contradictory regarding whether PI3K-Akt signaling exerts a positive or negative effect on AR. For instance, it was suggested that Akt and Mdm2 form a complex with AR and promote phosphorylation-dependent AR ubiquitination, targeting AR for degradation (58). In addition, it was shown that APPL (adapter protein containing PH domain, PTB domain, and leucine zipper motif), an Akt and PI3K catalytic subunit p110 interacting protein, may suppress AR activity via potentiating Akt activity (59).

We found that the PI3K-Akt cell survival pathway is critical for the growth of androgen-independent LNCaP-AI cells and is involved in the regulation of AR activity in an androgen-independent manner. p21^{WAF/CIP} is a target gene of PI3K-Akt signaling in prostate cancer cells. Given the anti-apoptotic activity of p21^{WAF/CIP}, our finding implicates that overexpression of p21^{WAF/CIP} may be a result of elevated PI3K-Akt signaling in androgen-independent prostate cancer cells, which provides a survival mechanism for prostate cancer cells and contributes to the development of androgen-independent growth and prostate cancer progression.

Acknowledgements

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