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5α-Androstane-3α,17β-Diol Supports Human Prostate Cancer Cell Survival and Proliferation Through Androgen Receptor-Independent Signaling Pathways: Implication of Androgen-Independent Prostate Cancer Progression

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Abstract Androgen and androgen receptor (AR) are involved in growth of normal prostate and development of prostatic diseases including prostate cancer. Androgen deprivation therapy is used for treating advanced prostate cancer. This therapeutic approach focuses on suppressing the accumulation of potent androgens, testosterone and 5α-dihydrotestosterone (5α-DHT), or inactivating the AR. Unfortunately, the majority of patients with prostate cancer eventually advance to androgen-independent states and no longer respond to the therapy. In addition to the potent androgens, 5α-androstane-3α,17β-diol (3α-diol), reduced from 5α-DHT through 3α-hydroxysteroid dehydrogenases (3α-HSDs), activated signaling may represent a novel pathway responsible for the progression to androgen-independent prostate cancer. Androgen sensitive human prostate cancer LNCaP cells were used to compare 5α-DHT and 3α-diol activated androgenic effects. In contrast to 5α-DHT, 3α-diol regulated unique patterns of β-catenin and Akt expression as well as Akt phosphorylation in parental and in AR-silenced LNCaP cells. More significantly, 3α-diol, but not 5α-DHT, supported AR-silenced LNCaP cells and AR negative prostate cancer PC-3 cell proliferation. 3α-diol-activated androgenic effects in prostate cells cannot be attributed to the accumulation of 5α-DHT, since 5α-DHT formation was not detected following 3α-diol administration. Potential accumulation of 3α-diol, as a result of elevated 3α-HSD expression in cancerous prostate, may continue to support prostate cancer growth in the presence of androgen deprivation. Future therapeutic strategies for treating advanced prostate cancer might need to target reductive 3α-HSD to block intraprostatic 3α-diol accumulation. J. Cell. Biochem. 104: 1612–1624, 2008.

Key words: androgen; androgen receptor; cell growth; prostate cancer

The human prostate is an androgen sensitive organ that depends on androgen for growth and development [Coffey and Isaacs, 1981]. Potent androgens such as testosterone and 5α-dihydrotestosterone (5α-DHT) as well as their corresponding nuclear receptor (androgen receptor; AR) have been heavily implicated in the development of normal prostate and prostatic diseases including prostate cancer [Voigt and Bartsch, 1986; Berger et al., 2004]. Current therapeutic strategies for treating patients with advanced prostate cancer, therefore, mainly rely on androgen deprivation [Sharifi et al., 2005] to suppress the intraprostatic accumulation of potent androgens or to inactivate AR

Abbreviations used: 3α-diol, 5α-androstane-3α, 17β-diol; 3α-HSD, 3α-hydroxysteroid dehydrogenase; 5α-DHT, 5α-dihydrotestosterone; AKR, aldo-keto reductase; AR, androgen receptor; ARG, androgen responsive gene; siRNA, small interfering RNA; TUNEL, terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling.

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trans-activation [Seidenfeld et al., 2000; Smith et al., 2004; Tay et al., 2004]. However, the vast majority of patients who initially respond to the therapy inevitably progress to highly aggressive, androgen-independent prostate cancer [Daneshgari and Crawford, 1993]. The mechanisms by which prostate cancer cells survive in androgen deprivation and progress to an androgen-independent state remain unclear.

The potent androgen 5α-DHT can be reduced to 5α-androstane-3α, 17β-diol (3α-diol) by a group of isozymes named 3α-hydroxysteroid dehydrogenases (3α-HSDs) in the prostate. Two 3α-HSD isozymes, type 2 3α-HSD (AKR1C3) and type 3 3α-HSD (AKR1C2), have been identified in the human prostate [Penning et al., 2000]. Both 3α-HSDs possess dominant 5α-DHT reduction activity toward 3α-diol formation [Lin et al., 1997; Rizner et al., 2003; Penning et al., 2007]. In addition, elevated levels of 3α-HSD expression have been demonstrated in primary cultures of prostate epithelial cells derived from prostate cancer [Lin et al., 1997; Rizner et al., 2003] and in prostate cancer tissues [Nakamura et al., 2005; Fung et al., 2006; Stanbrough et al., 2006]. These results strongly suggest that 3α-diol can be accumulated in cancerous prostate.

Based on 3α-diol's low affinity toward the AR, 3α-diol has been recognized as a weak androgen and does not have androgenic effects [Bauman et al., 2006]. However, this assumption does not explain why 3α-diol is more potent than 5α-DHT or testosterone when inducing prostatic hyperplasia in castrated dogs [Walsh and Wilson, 1976; Jacobi et al., 1978]. 3α-diol is also an active androgen in virilizing the urogenital tract of female rat embryos [Schultz and Wilson, 1974], plays a significant role in murine parturition [Mahendroo et al., 1996], and is implicated in prostate and penile development of female fetuses of marsupials [Shaw et al., 2000; Leihy et al., 2004]. In addition, Agapova et al. [2006] demonstrated that 3α-diol is responsible for optic nerve head astrocyte mobility and survival. Growing evidence suggests that 3α-diol might be an important androgen with its own functions in androgen target tissues.

The mechanism of 3α-diol-activated androgenic effects remains undefined. Ding et al. [1998] demonstrated that 3α-diol forms a complex with sex hormone-binding globulin (SHBG) at the cell surface of androgen target tissues to stimulate rapid accumulation of intracellular cAMP with subsequent activation of the AR. However, 3α-diol-activated signaling may be different from the classical AR-mediated signaling pathway in prostate cells. Nunlist et al. [2004] reported that 3α-diol is as potent as 5α-DHT in stimulating androgen sensitive human prostate cancer LNcaP cell proliferation, but does not execute similar levels of AR *trans*-activation. In addition, 3α-diol-regulated genes can be distinguished from 5α-DHT-regulated androgen responsive genes (ARGs) [Zimmerman et al., 2004]. The present study emphasizes that 3α-diol can activate AR-independent ARG expression and cytoplasmic signaling, as well as promote prostate cell survival and proliferation. These results suggest that potential intraprostatic accumulation of 3α-diol through elevated 3α-HSD expression in cancerous prostate might activate cell survival pathway and support androgen-independent cancer progression in the presence of androgen deprivation therapy.

MATERIALS AND METHODS

Materials

RPMI 1640 medium, F-12 nutrient mixture, OPTI-MEM, penicillin-streptomycin, and fetal bovine serum (FBS) were purchased from Invitrogen (Grand Island, NY). Charcoal-dextran treated (CD) FBS with testosterone $<10^{-10}$ M was obtained from HyClone (Logan, UT). Presynthesized AR-specific and control SMARTpools® small interfering RNA (siRNA) duplexes were obtained from Upstate (Charlottesville, VA). Mouse anti-human AR monoclonal antibody was obtained from Novocastra (UK). Rabbit anti-Akt (or protein kinase B; PKB) antibody, rabbit anti-phospho-Akt Ser(473) antibody, and rabbit anti-β-catenin antibody were purchased from Cell Signaling Technology (Danvers, MA). Mouse anti-β-actin antibody was obtained from Sigma (St. Louis, MO). 3α-diol and 5α-DHT were obtained from Steraloids (Newport, RI).

Plasmid Constructs

Plasmid-based AR-specific and nonspecific control siRNA constructs were modified from our previous report with the excision of the IRES sequence [Yang et al., 2005b]. These constructs were designated as pSiAR-EGFP and pSiCon-EGFP for AR-specific and control siRNA plasmids, respectively. These siRNA

plasmids contain enhanced green fluorescence protein (EGFP) driven by the cytomegalovirus (CMV) promoter. Transfected cells constitutively expressing EGFP allowed for rapid enrichment.

Human Prostate Cell Culture and Transfection

Androgen sensitive human metastatic prostate adenocarcinoma LNCaP cells (CRL-1740) and human bone metastasized prostate cancer PC-3 cells (CRL-1435) were obtained from ATCC (Manassas, VA). LNCaP cells were maintained in a complete growth medium (RPMI1640 supplemented with 10% FBS) [Nunlist et al., 2004] and used at low passage numbers (less than 35 passages in total). PC-3 cells were maintained in F-12 nutrient mixture containing 7% FBS.

Suppression of the endogenous AR expression in LNCaP cells was achieved using two siRNA approaches: an oligo-based siRNA duplex and a plasmid-based siRNA construct. The oligo-based SMARTpools® siRNA duplex was used to analyze androgen-stimulated ARG expression and cytoplasmic signal activation based on its high transfection efficiency. Briefly, trypsinized LNCaP cells (2×10^6) were mixed with 20 μM either SMARTpools® AR-specific or control siRNA duplex in 100 μl of Nucleofector solution R (Amaxa Biosystems, Gaithersburg, MD); and transfection was completed with the Nucleofector device (Amaxa Biosystems). Cells were then returned to their growth medium in 60 mm tissue culture plates for 24 h before serum deprivation (OPTI-MEM plus 2% CD FBS) and androgen stimulation.

The siRNA plasmid was used to determine androgen-stimulated cell proliferation and survival based on its sustained suppression of AR expression in prostate cells. Briefly, LNCaP cells were transfected with either pSiAR-EGFP or pSiCon-EGFP plasmid with Lipofectamine 2000 (Invitrogen) using our reported procedures [Yang et al., 2005b]. Cells were then returned to their growth medium with or without androgen supplementation at 4 h following transfection.

Androgen Stimulation

To determine androgen conversion, LNCaP cells (5×10^5) were seeded in the growth medium in six-well tissue culture plates for adherence. Cells were subjected to serum deprivation for 24 h and switched to 1 ml serum free OPTI-MEM medium for another 4 h. Cells were left untreated or treated with either 10^{-8} M 5α-DHT (positive control) or 3α-diol. Samples were collected at 24 h following androgen treatment by harvesting the cells and media together, immediately frozen with dry ice in acetone, and stored at −80°C until analysis.

To study androgens-stimulated ARG expression and Akt phorphorylation, parental and siRNA duplex transfected LNCaP cells (1×10^6) were first seeded in 60 mm tissue culture plates for adherence. Cells were then subjected to serum deprivation for 24 h followed by 10^{-8} M androgen stimulation.

Androgen-stimulated prostate cell proliferation was performed in both LNCaP and PC-3 cells. At 24 h following pSiAR-EGFP or pSiCon-EGFP transfection, EGFP expressing LNCaP cells (1,000) were directly distributed into each well of a 96-well tissue culture plate containing 200 μl of their growth medium with or without androgens using MoStar cell sorter [Yang et al., 2005b]. PC-3 cells (1,000 cells in 100 μl) were also seeded in each well of a 96-well plate in their growth medium, and were either left untreated or treated with 3α-diol. For cell death analysis, siRNA plasmid transfected LNCaP cells were either left untreated or treated with androgens.

5α-DHT Measurement

LC/MS/MS detection of 5α-DHT was performed as described [Titus et al., 2005] with modifications. Deuterated testosterone and 5α-DHT (1 ng each) were added to thawed LNCaP cells with media as internal standards; and samples were extracted three times with 1 ml of 9:1 chloroform/acetone. Collected organics were evaporated under vacuum and analytes concentrated using solid phase extraction cartridges (SPEC-C18AR, Varian) conditioned with methanol and water. Samples were applied in 1:4 methanol/water. Samples were eluted in methanol, dried under vacuum, and reconstituted in 65% methanol for analysis.

The LC/MS/MS system used was the Thermo Finnnagan Surveyor quaternary MS pump, solvent degasser, and a Surveyor auto sampler interfaced with TSQ Quantum ULTRA mass spectrometer. A Phenomenex Luna C18 (3 μm, 150 × 2.0 mm) column was used to separate testosterone and 5α-DHT. The column was

maintained at 30°C. An HPLC gradient profile was used from 65% to 75% methanol in 2.25 min followed: 60% B from 0.0 to 1.0 min, 70% to 100% B from 1.1 to 9.0 min, 100% B from 9.0 to 14.0 min, 100–60% B from 14.0 to 14.5 min. The column was equilibrated at 60% B for 12 min prior to sample injection.

Testosterone and 5α-DHT were ionized using an atmospheric pressure chemical ionization source in positive ion mode and were quantified using unique product ions. The parent/product ion pairs of *m/z* 289.2 to 97.0 for testosterone, *m/z* 292.2 to 97.0 for internal standard testosterone-d_3, *m/z* 291.2 to 255.1 for 5α-DHT, and *m/z* 294.2 to 258.1 for internal standard 5α-DHT-d_3 were monitored and the product ion was used to quantify androgens. Optimized collision energy settings for testosterone, testosterone-d_3 and 5α-DHT, 5α-DHT-d_3 were 25, 18 V, respectively. Other mass spectrometry parameters were collision gas pressure 1.2 m Torr, discharge current 4.0 kV, vaporizing temperature 480°C, sheath gas 30 Arb, Auxiliary gas 7 Arb, capillary temperature 280°C. Nitrogen was used for all gas inputs.

Western Blot Analysis

To determine levels of target protein expression, total cellular proteins were isolated by lysing cells with RIPA buffer that was supplemented with 0.1 mM phenylmethylsulphonylfluoride (PMSF) and a proteinase inhibitor cocktail (Roche, Indianapolis, IN) [Yang et al., 2005b]. Protein concentrations were determined by a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL). Aliquots of 30 μg of the cellular proteins were separated on 10% Tris–HCl gel (Bio-Rad, Hercules, CA); and proteins were transferred to PVDF membranes (Bio-Rad). Detection of the AR and β-actin expression was performed as reported [Yang et al., 2005b]. β-catenin, total Akt, and phospho-Akt expression were detected by incubating the PVDF membranes with primary antibodies against these molecules followed by appropriate peroxidase-conjugated secondary antibody incubation. Immunoreactive proteins were detected using an enhanced chemiluminescent (ECL) reagent (Pierce). To quantify levels of the target protein expression, images of immunoreactive bands were captured; and intensities of these bands were calculated using the computerized Quantity One® image analysis software (Bio-Rad).

Cell Proliferation Assay

Cell proliferation was performed directly in 96-well tissue culture plates using a XTT cell proliferation assay kit (Roche). Briefly, an aliquot of 50 μl XTT labeling mixture was added to each well of the 96-well plates containing 100 μl of culture medium and incubated at 37°C for 4 h. Absorbance was obtained by scanning the plates using μQuant microplate reader (Bio-Tek, Winooski, VT). Data normalization and analysis was performed as previously reported [Nunlist et al., 2004].

Terminal Deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling (TUNEL) Analysis

TUNEL analysis was performed using IHC-like staining procedures in cell block sections. Briefly, on day 6 after siRNA plasmid transfection and androgen stimulation, LNCaP cells were trypsinized and collected through centrifugation. Cell pellets were fixed in 10% formalin, immersed in agarose, and subjected to paraffin embedding. The cell blocks were sectioned, dewaxed, and rehydrated [Yang et al., 2005b]. Cells were permeabilized by incubating the sections with 20 μg/ml proteinase K at room temperature for 20 min; and DNA fragmentation was detected using an in situ Cell Death Detection kit (Roche). Positive control was prepared by digesting parental LNCaP cell sections with 1 U/μl DNase I at room temperature for 10 min. Following the terminal deoxynucleotidyl transferase reaction, cell sections were incubated with the converter-alkaline phosphotase. A Fast Red substrate was added to the slides and incubated at room temperature for an additional 10 min for color development. Slides were then washed and sealed with an aqueous mounting medium. Apoptotic cells with damaged DNA were stained positive with a bright red color.

To quantify the number of apoptotic cells, five sections from each experimental group were studied. On each slide, three fields were randomly picked; and images were taken at 200× magnification by Olympus BX51 microscope equipped with SPOT Insight CCD digital camera and SPOT Advance software (Diagnostic Instruments; Sterling Heights, MI). Total number of cells and red cells were counted for each image. The mean and standard deviation of the percentage of TUNEL positive cells for each experimental group were calculated.

Statistical Analysis

Statistical differences between two experimental groups were evaluated using student *t*-test. Statistically significant difference was set when $P < 0.01$.

RESULTS

3α-Diol and 5α-DHT Induced Differential ARG Expression and Cytoplasmic Signaling

Nunlist et al. [2004] reported that β-catenin and Akt respond to both 3α-diol and 5α-DHT stimulation in LNCaP cells; but temporal regulation of steady state levels of β-catenin and Akt mRNA expression was different between 3α-diol and 5α-DHT treatments. Western blot analysis confirmed that in spite of initial (within 30 min) suppression of β-catenin expression by both androgens, 5α-DHT-elevated β-catenin expression was almost immediate and sustained for 24 h. In contrast, recovery of initial 3α-diol-suppressed β-catenin expression was slower and extended to 8 h (Fig. 1A,B); and levels of β-catenin in 3α-diol-treated LNCaP cells were doubled as compared to untreated control at 24 h. In addition, 5α-DHT-stimulated Akt protein expression peaked at 30 min but gradually subsided (Fig. 2A,B). On the contrary, 3α-diol suppressed Akt expression between 30 min and 4 h; but elevated Akt expression was detected at 8 h post 3α-diol treatment (Fig. 2A,B).

5α-DHT regulated Akt phosphorylaiton in a time-dependent manner. However, 3α-diol had more potent and sustained effects on Akt phosphorylation in LNCaP cells as compared to the same concentration of 5α-DHT (Fig. 2A). Semi-quantitative analysis of Akt phosphorylation levels demonstrated that 3α-diol induces

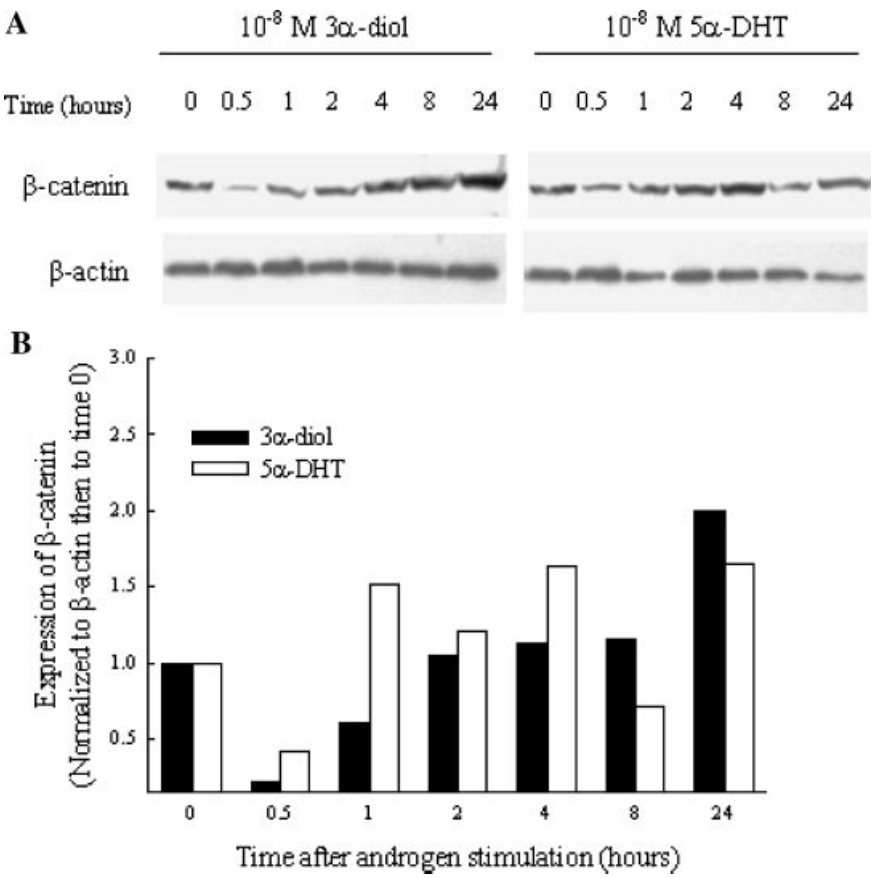

Fig. 1. Temporal regulation of β-catenin expression by 3α-diol and 5α-DHT in LNCaP cells. **A**: Western blot analysis of androgen-regulated β-catenin expression between 30 min and 24 h in LNCaP cells. **B**: Semi-quantitative levels of β-catenin expression between 3α-diol and 5α-DHT treated LNCaP cells. Intensities of immunoreactive bands were first normalized to levels of β-actin within each time point; and levels of the normalized values were then adjusted to untreated cells for each androgen treatment. Experiments were repeated at least twice and a representative result was presented.

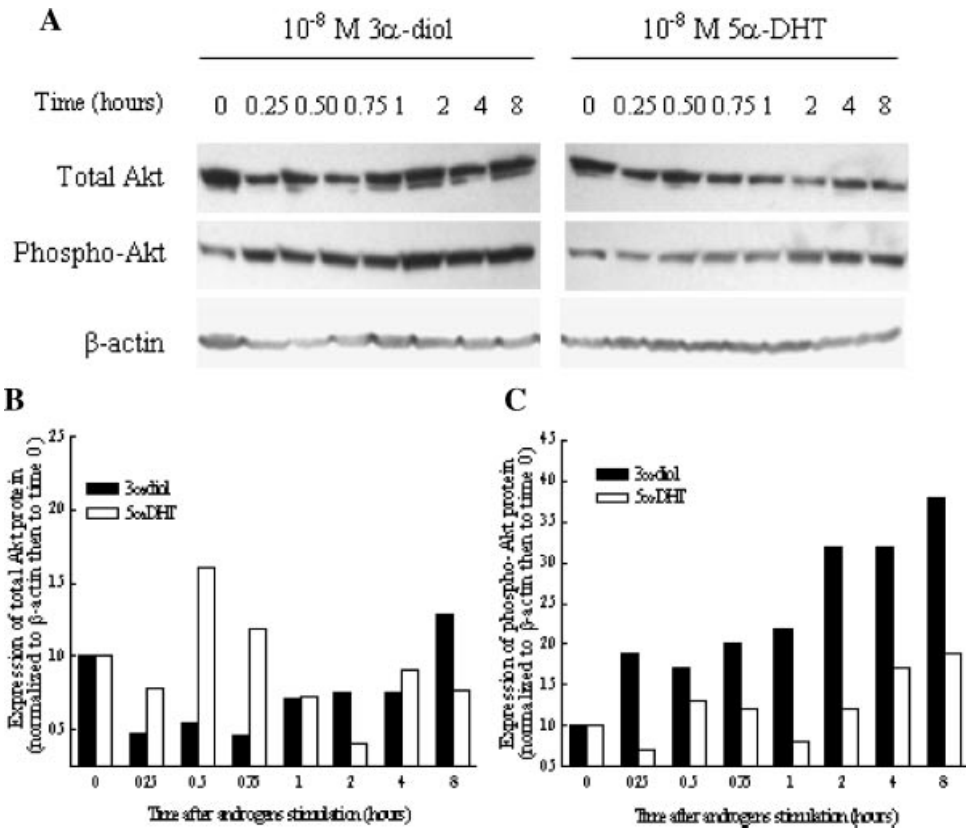

Fig. 2. Temporal regulation of Akt expression and phosphorylation in androgen-stimulated LNCaP cells. **A**: Western blot analysis was performed using total cellular proteins obtained from either 3α-diol or 5α-DHT treated LNCaP cells. **B**: Semi-quantitative presentation of Akt expression was first normalized to β-actin within each time point and then adjusted to normalized values of untreated cells. **C**: Semi-quantitative levels of Akt phosphorylation. Experiments were repeated at least twice and a representative result was presented.

Akt phosphorylation by about twice as much as 5α-DHT treatment at all time points (Fig. 2C).

AR-Specific siRNA Duplex Suppressed AR Expression

To investigate the involvement of the AR in androgen actions, RNA interference (RNAi) technology was introduced to suppress endogenous AR expression in LNCaP cells. To determine the efficiency of an AR-specific siRNA duplex in suppressing AR expression, LNCaP cells were transfected with an AR-specific or control *SMART*pools® siRNA duplex. Western blot analysis demonstrated that the AR-specific siRNA duplex successfully silences AR expression in LNCaP cells between 24 and 72 h following transfection (Fig. 3). Although a higher transfection efficiency was achieved using the duplex siRNA as compared to plasmid-based siRNA construct, gene silencing by the duplex siRNA was transient and the AR re-expressed in LNCaP cells after 72 h post-transfection (data not shown). As demonstrated by Yang et al. [2005b] AR protein expression can be suppressed in LNCaP cells transfected with an AR-specific siRNA plasmid; and the suppressed AR expression can be sustained for at least 6 days.

5α-DHT Accumulation in 3α-Diol-Treated Prostate Cells

Limits of detection for testosterone and 5α-DHT using LC/MS/MS were 8.7 and 43 fmol, respectively. 5α-DHT was only detected in positive control samples at 0.4 nM but not in 3α-diol-treated LNCaP cells or negative control samples. However, testosterone was detected in all samples but was below the limit of quantification.

3α-Diol Activated AR-Independent ARG Expression and Akt Signaling

Transfection of nonspecific siRNA duplex did not alter androgen-regulated ARG expression

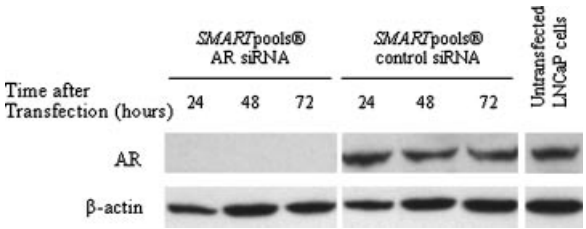

Fig. 3. Suppression of AR expression using the SMARTpools® siRNA duplex. Western blot analysis was used to determine the successful suppression of the AR in LNCaP cells. There was no detectible AR protein expression in LNCaP cells between 24 and 72 h following the AR-specific siRNA duplex transfection. In contrast, levels of AR expression were not affected in cells transfected with the control siRNA duplex.

or Akt phosphorylation as compared to untransfected parental LNCaP cells (data not shown). 3α-diol continued to regulate both β-catenin and Akt protein expression in a temporal manner in AR-silenced LNCaP cells (Fig. 4). 3α-diol-regulated β-catenin expression exhibited similar temporal changes with and without the AR; levels of β-catenin accumulation were suppressed at 30 min but elevated at 24 h following 3α-diol stimulation. In the absence of the AR, levels of total Akt expression were highly elevated between 1 and 24 h and peaked at 4 h following 3α-diol treatment (Fig. 4A,B). 3α-diol was also capable of stimulating Akt phosphorylation in LNCaP cells in an AR-independent manner; a twofold induction in Akt phosphorylation levels was observed at 24 h after 3α-diol treatment in AR-silenced LNCaP cells (Fig. 4A,B).

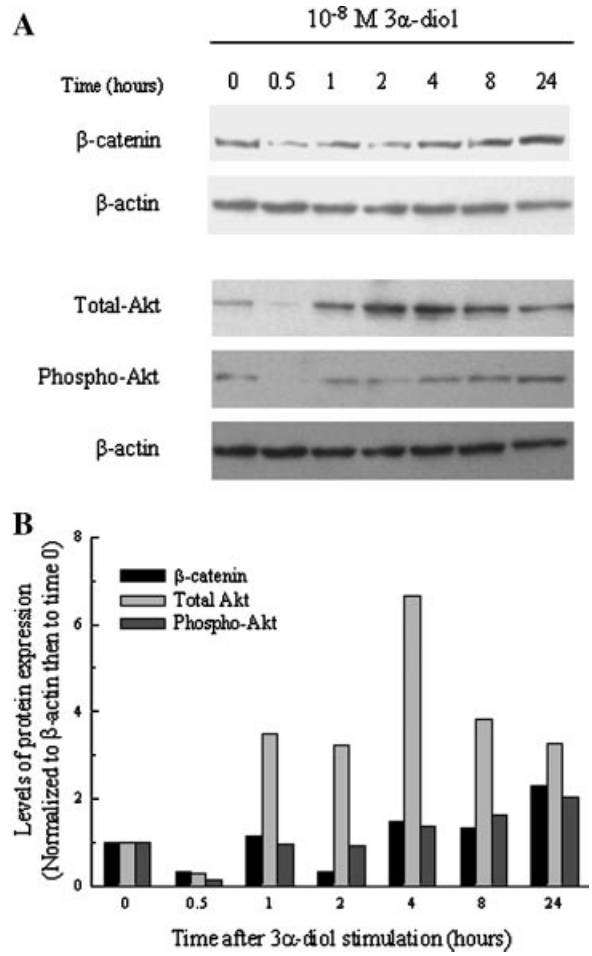

Fig. 4. Temporal regulation of 3α-diol-stimulated ARG expression and Akt phosphorylation in AR-silenced LNCaP cells. **A**: Levels of β-catenin and Akt expression as well as Akt phosphorylation were determined in LNCaP cells following the AR-specific siRNA duplex transfection and 3α-diol stimulation using Western blot analysis. **B**: Semi-quantitative presentation of 3α-diol-regulated ARG expression and Akt phosphorylation was performed by first normalizing to levels of β-actin expression and then to untreated controls. Experiments were repeated at twice and a representative result was presented.

3α-Diol Promoted Prostate Cell Proliferation and Survival

Similar to reports by others, LNCaP cells required a functional AR for their proliferation in their growth medium (Fig. 5A). Supplementation of 5α-DHT did not support AR-silenced LNCaP cell proliferation (Fig. 5A). In contrast, the addition of 3α-diol partially restored LNCaP cell proliferation in the absence of the AR. Cell number increased by 4.2- and 6.4-fold at 11 and 14 days in 3α-diol-treated AR-silenced LNCaP cells as compared to 1.2- and 1.7-fold increases in untreated cells during the same time periods. However, 3α-diol-stimulated AR-silenced LNCaP cell proliferation was not as rapid as pSiCon-EGFP-transfected LNCaP cells during the entire course of the experiments (Fig. 5A). In addition, 3α-diol significantly promoted AR negative PC-3 cell proliferation as compared to untreated cells in their growth medium (Fig. 5B).

The lack of cell proliferation in the AR-silenced LNCaP cells might result from elevated cell death. AR silencing in LNCaP cells resulted in apoptosis as demonstrated by an increased number of TUNEL positive cells compared to control siRNA plasmid-transfected LNCaP cells (Fig. 6 and Table I). 5α-DHT supplementation in the complete growth medium did not reduce the number of TUNEL positive cells (data not shown). In contrast, 3α-diol-treated AR-silenced LNCaP cells had significantly reduced number

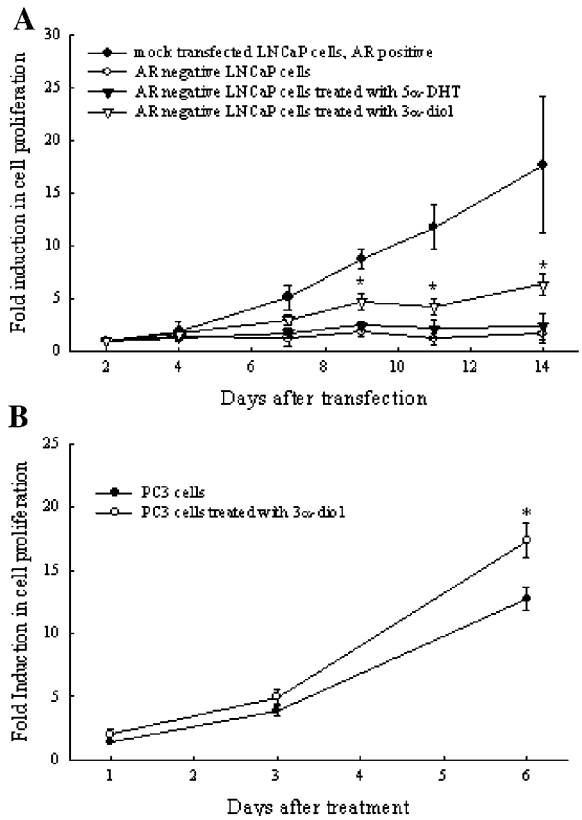

Fig. 5. 3α-diol-supported prostate cell proliferation. **A**: LNCaP cell proliferation in the absence of the AR. LNCaP cells were transfected with the AR-specific or nonspecific siRNA plasmid. The transfected cells were left untreated or treated with either 3α-diol or 5α-DHT in their growth medium and sorted into 96-well tissue culture plates for cell proliferation assay. Number of viable cells was determined at designated time points using the XTT cell proliferation assay kit. **B**: 3α-diol-stimulated PC-3 cell proliferation. Human prostate cancer PC-3 cells were seeded in 96-well tissue culture plates in their growth medium followed by 3α-diol treatment. Cell number was determined by the XTT assay for a period of 6 days. Asterisk (*) indicates statistical significance between untreated and 3α-diol-treated cells ($P < 0.01$). All experiments were prepared in triplicate; and data were presented as mean ± standard error of means (SEM) from at least three independent experiments.

of TUNEL positive cells as compared to untreated AR-silenced LNCaP cells (Table I).

DISCUSSION

This study investigated whether or not 3α-diol-activated signaling can be distinguished from the classical AR-mediated pathway, and whether or not 3α-diol can activate cell survival signaling in prostate cells. Our results demonstrated that 3α-diol can have unique androgenic effects on ARG expression and cytoplasmic signaling independent from the AR pathway. More importantly, in contrast to 5α-DHT, 3α-diol can support human prostate cancer cell survival and proliferation in the absence of the AR.

3α-diol has been generally classified as a weak androgen based on its low affinity toward the AR. Therefore, 3α-diol-mediated androgenic effects have been attributed to its oxidized product, 5α-DHT, through oxidative 3α-HSDs [Bauman et al., 2006]. This pathway is supported by the evidence that 3α-diol's androgenic effects in prostate bud formation of female Tammar Wallaby pouch young can be blocked by the administration of antiandrogens [Leihy et al., 2001]. However, if 3α-diol needs to be oxidized to 5α-DHT to exert its androgenic effects, it is difficult to explain why 3α-diol stimulates robust LNCaP cell proliferation [Nunlist et al., 2004] without detectible 5α-DHT accumulation [Rizner et al., 2003].

Mechanisms that are responsible for 3α-diol to exert its androgenic effects remain undefined. Based on gene expression profiling analysis, 3α-diol responsive genes are different from 5α-DHT responsive genes in LNCaP cells [Nunlist et al., 2004; Zimmerman et al., 2004]. β-catenin and Akt were two of the ARGs whose steady state levels of mRNA are differentially regulated by 3α-diol and 5α-DHT. β-catenin is a multifunctional protein involved in two apparently independent processes, cell–cell adhesion and signal transduction, through *Wnt* signaling pathway [Aguilera et al., 2007]. The canonical *Wnt* signaling pathway modulates androgen signaling at multiple levels; and β-catenin represents a major molecule associated with the AR [Sharma et al., 2002]. The phosphoinositide 3 kinase (PI3K)/Akt signaling is recognized as a major cell proliferation and survival pathway in a variety of cells [Chinni and Sarkar, 2002; Chang et al., 2003]. Both β-catenin and Akt signaling pathways have been implicated in the development of androgen-independent prostate cancer [Terry et al., 2006; Xin et al., 2006; Wu and Huang, 2007].

To compare 3α-diol- and 5α-DHT-activated androgenic effects in LNCaP cells, our results confirmed that β-catenin and Akt protein expression as well as Akt phosphorylation are differentially regulated by both androgens. Consistent with published results [Sun et al., 2003; Kang et al., 2004; Gatson et al., 2006], 5α-DHT activates nongenomic signaling such as

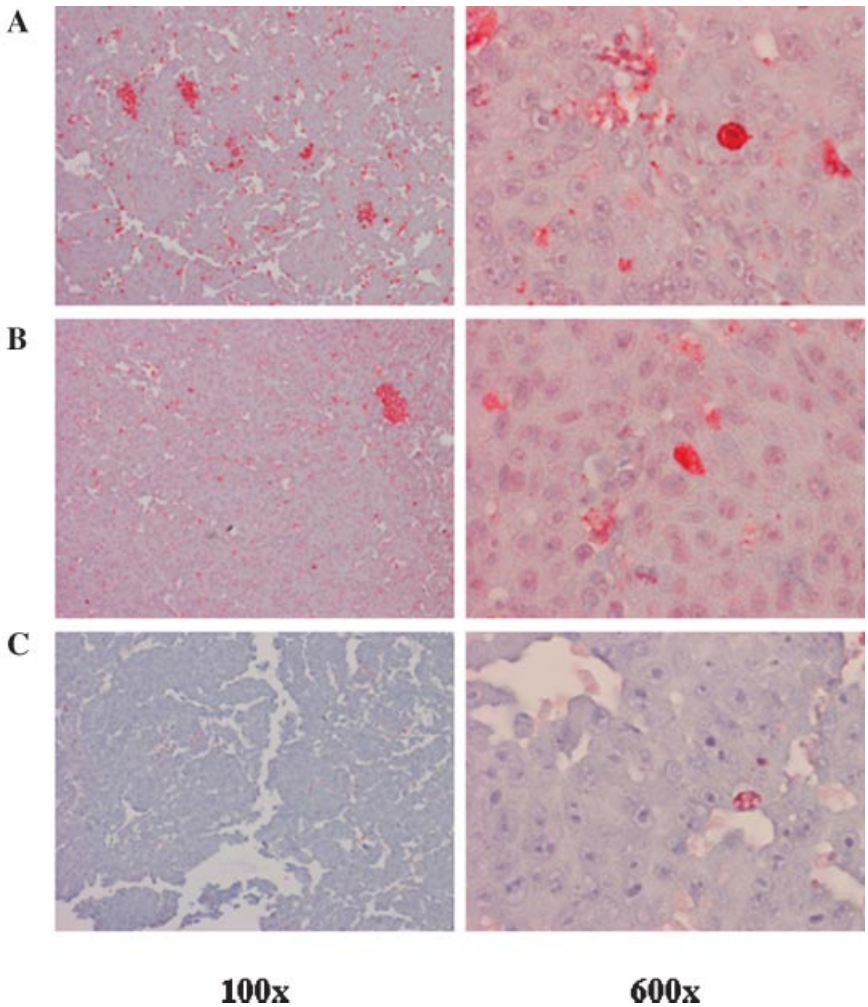

Fig. 6. Evidence of 3α-diol-induced LNCaP cell survival in the absence of the AR. Apoptosis was determined in AR-specific and control siRNA plasmid transfected LNCaP cells. The transfected cells were then treated with or without 3α-diol. **A**: LNCaP cells transfected with the pSiAR-EGFP plasmid were incubated in their growth medium. **B**: pSiAR-EGFP transfected LNCaP cells were maintained in the growth medium supplemented with 10^{-8} M 3α-diol. **C**: LNCaP cells transfected with nonspecific pSiCon-EGFP plasmid were cultured in the growth medium.

Akt phosphorylation in LNCaP cells. The lower levels of Akt phosphorylation following 5α-DHT stimulation compared to other reports may reflect the passage-dependent PI3K/Akt activation in LNCaP cells [Lin et al., 2003]. If 3α-diol needs to be oxidized to 5α-DHT before it can exert its androgenic actions in prostate cells, 3α-diol-regulated ARG expression and Akt

TABLE I. Quantification of 3α-Diol Effects on Apoptosis in AR-Silenced LNCaP Cells

Plasmid siRNA transfection	TUNEL positive cells (% mean ± SD)	Transfection efficiency (%)
pSiAR-EGFP	$15.57 \pm 1.15^{a,b}$	13.29
pSiAR-EGFP treated with 3α-diol	7.10 ± 1.54^{a}	13.50
pSiCon-EGFP	4.11 ± 1.06	13.85

[a]The percentages of TUNEL positive cells were significant higher in pSiAR-EGFP-transfected LNCaP cells, with or without 3α-diol supplementation, than in pSiCon-EGFP-transfected cells ($P < 0.01$).
[b]The percentages of TUNEL positive cells in pSiAR-EGFP-transfected LNCaP cells were significant higher than their counterparts treated with 10^{-8} M 3α-diol ($P < 0.01$).

activation would follow the temporal changes of 5α-DHT-activated responses. However, our results showed that 3α-diol-regulated Akt expression peaks at 8 h whereas 5α-DHT-regulated Akt expression peaks at 30 min. More significantly, 3α-diol activated more rapid and robust Akt phorphorylation as compared to 5α-DHT stimulation. 3α-diol-regulated Akt expression and phosphorylation cannot be attributed to 5α-DHT formation, since 5α-DHT was not detected in LNCaP cells following 3α-diol administration. Such observations were consistent with results reported by Agapova et al. [2006] in which 3α-diol is shown to be more potent than a synthetic 5α-DHT agonist in stimulating PI3K/Akt signaling in optic nerve head astrocytes.

To evaluate the involvement of the AR in 3α-diol-activated androgenic effects, AR expression in LNCaP cells was suppressed using siRNA approaches. Despite the observations that the AR-specific siRNA duplex only transiently suppresses AR expression in LNCaP cells, the nearly completed suppression of AR expression was appropriate for gene expression and signal transduction studies. Levels of 3α-diol-regulated β-catenin expression were similar between cells with and without the AR suggesting that 3α-diol-regulated β-catenin expression can be independent from the AR. Patterns of the 3α-diol-stimulated Akt expression and phosphorylation were different in the presence and the absence of the AR; and the results might reflect a reciprocal interaction between the AR and PI3K/Akt signaling pathways in LNCaP cells [Lin et al., 2003]. These results, together with metabolism studies, suggest that 3α-diol-activated ARG expression and cytoplasmic signaling can be independent of 5α-DHT and the AR. 3α-diol, therefore, must activate alternative pathways other than the AR to execute its androgenic actions.

Suppression of AR expression or disruption of AR signaling has been shown to suppress cell proliferation [Chen et al., 1998; Eder et al., 2000; Wright et al., 2003] or to induce cell death [Yang et al., 2005b]. Supplementation of 5α-DHT did not support AR-silenced LNCaP cell proliferation. This is consistent with the concept that 5α-DHT utilizes AR pathway to transduce its androgenic signals. In contrast, 3α-diol supported LNCaP cell proliferation in the absence of the AR, and stimulated AR negative PC-3 cell proliferation. 3α-diol-stimulated PC-3 cell proliferation cannot be attributed to 5α-DHT or the AR since 3α-diol is not oxidized to 5α-DHT in PC-3 cells [Rizner et al., 2003] and 5α-DHT suppresses cell proliferation in PC-3 cells stably transfected with the AR [Yuan et al., 1993]. The 3α-diol-stimulated AR-silenced LNCaP cell proliferation might result from reduced cell death. However, 3α-diol-activated LNCaP cell survival pathways must work with serum components since supplementation of 3α-diol in a reduced serum conditioin (OPTI-MEM plus 2% CD FBS) did not support cell proliferation (data not shown). Consistent with other reports that PI3K/Akt signaling is a dominant survival pathway in LNCaP cells [Lin et al., 1999; Sun et al., 2003; Yang et al., 2005a], the inclusion of a PI3K inhibitor, LY294002, suppressed cell survival in both 3α-diol and 5α-DHT-treated LNCaP cells (data not shown). Although it is still unclear which mechanism that 3α-diol uses to rescue cell death and promote cell proliferation, 3α-diol-activated, AR-independent prostate cell survival and proliferation may result from Akt and/or β-catenin activation (Fig. 7), since elevated PI3K/Akt signaling through a variety of stimuli can contribute to the failure of androgen deprivation therapy [Murillo et al., 2001; Lin et al., 2003].

Testosterone is synthesized from the testes and the adrenal glands, and converted to related androgen metabolites in the prostate using tissue-specific steroidogenic enzymes. Androgen deprivation therapy is intended to block potent androgen accumulations. However, levels of potent androgens remain relatively constant in the prostate before and after the therapy [Nishiyama et al., 2004; Titus et al., 2005]. Abnormal intraprostatic androgen metabolism and androgen metabolite accumulation can result from deregulated androgen metabolizing enzyme expression [Mizokami et al., 2004]. There are two major 3α-HSD isozymes, AKR1C2 and AKR1C3, in the human prostate [Lin et al., 1997; Penning et al., 2000]. Both isozymes catalyze mainly 5α-DHT reduction for 3α-diol formation [Lin et al., 1997; Rizner et al., 2003]. Elevated expression of 3α-HSDs has been observed in primary cultures of human prostate cancer cells as compared to those derived from normal prostate [Lin et al., 1997; Rizner et al., 2003], and confirmed in localized and advanced prostate cancer tissues [Nakamura et al., 2005; Fung et al., 2006;

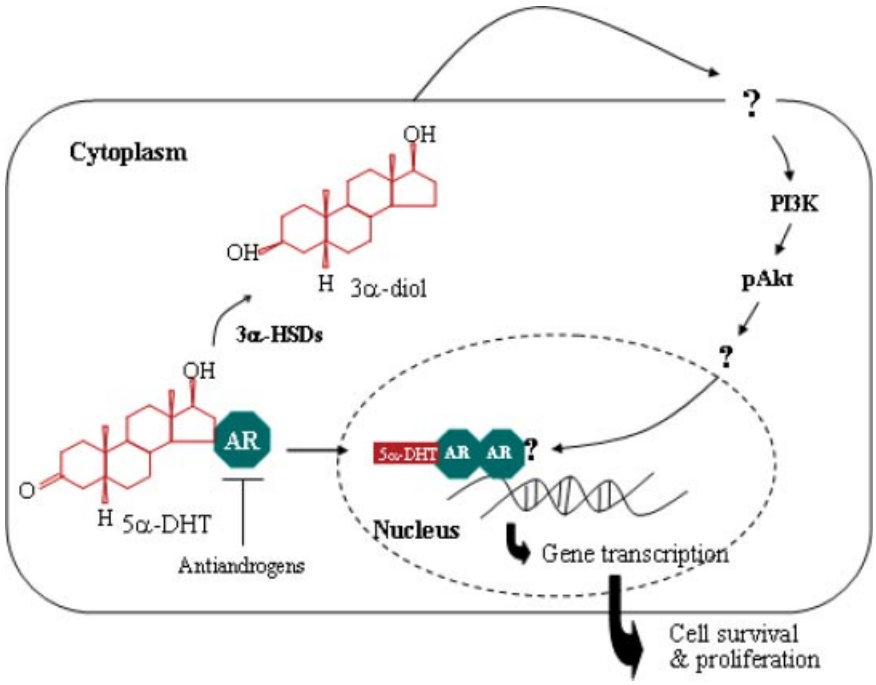

Fig. 7. Proposed mechanism of 3α-diol-activated androgenic actions. Instead of being oxidized to 5α-DHT to activate AR pathway, 3α-diol activates cytoplasmic Akt signaling pathway through an AR-independent manner. This 3α-diol-activated androgenic actions may be responsible for continued prostate cancer cell growth in the presence of androgen deprivation therapy.

Stanbrough et al., 2006]. Based on this study, levels of intraprostatic 3α-diol need to be determined in order to establish roles of 3α-HSDs and 3α-diol in prostate cancer development and progression. Future therapeutic development for treating advanced prostate cancer might need to target reductive 3α-HSDs to suppress intraprostatic accumulation of 3α-diol in conjunction to current androgen deprivation therapy.

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