Dehydroepiandrosterone-induced proliferation of prostatic epithelial cell is mediated by NFKB via PI3K/AKT signaling pathway

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Abstract

Dehydroepiandrosterone (DHEA) is an endogenous steroid that is metabolized to androgens and/or estrogens in the human prostate. DHEA levels decline with age, and use of DHEA supplements to retard the aging process is of unproved effectiveness and safety. In this study, rat ventral prostatic epithelial cells were used to determine whether DHEA-modulated proliferation and prostate-specific antigen (PSA listed as KLKB1 in the MGI Database) production were mediated via the androgen receptor (AR) and its potential mechanism. We demonstrated that proliferation of prostatic epithelial cells and increase of PSA expression induced by DHEA were neutralized by Casodex or *Ar* siRNA, two specific AR blockers. DHEA stimulated *Nfkb* DNA binding activity, with this effect being blunted by Casodex or *Ar* siRNA. Moreover, the inhibition of the phosphatidylinositol 3-kinase (PI3K)/AKT nullified the effects of DHEA on NFKB activation. These findings suggested that DHEA stimulated normal prostatic epithelial cell proliferation, and AR is involved in DHEA-induced PSA expression in normal prostatic epithelial cells. This stimulation effect induced by DHEA is mediated by the activation of NFKB via PI3K/AKT pathway.
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Introduction

Dehydroepiandrosterone (DHEA) and its sulfated form DHEA-S are the most abundant steroids in humans, produced by the adrenal cortex (Labrie *et al.* 2001). The secretion of DHEA and DHEA-S by the adrenals increases during the adrenarche in children at the age of 6–8 years, and elevated values of circulating DHEA-S and DHEA are maintained throughout adult life, providing high levels of substrates for conversion into potent androgens and estrogens in peripheral tissues (Labrie *et al.* 1998). In the US, DHEA is widely available as an over-the-counter dietary supplement, and is increasingly self-prescribed for its alleged anabolic and anti-aging effects, with unsubstantiated claims of beneficial effects as well as uncertain long-term safety (Alesci *et al.* 2005). In aged adults, the use of DHEA as a dietary supplement is of potential concern in that its androgenic or estrogenic actions may stimulate proliferation and other adverse effects in cancer cells within the prostate or breast (Arnold *et al.* 2005). DHEA is an important source of both androgenic and estrogenic ligands in the human prostate (Arnold 2009). DHEA-S is present in high levels in the prostate, as is the sulfatase that converts DHEA-S to DHEA (Klein *et al.* 1989). Receptors for DHEA or DHEA-S have not been definitively isolated (Widstrom & Dillon 2004). It was reported that DHEA acts on the direct agonistic and antagonistic effects on androgen receptor (AR), estrogen receptor (ER or ESR), and ERβ (ESR2) in human prostate cancer (PCa) and other cells (Chen *et al.* 2005).

AR is a member of the steroid receptor superfamily and is a nuclear transcription factor. Upon binding to AR, androgen activates AR, which, in turn, interacts with androgen response elements (ARE) in the promoter of target genes including prostate-specific antigen (PSA), regulating the transcription of target genes. It has been known that there are NFKB binding sites in the promoter of AR (Zhang *et al.* 2004), suggesting that NFKB may regulate the expression of AR. The activation of AKT and NFKB has been involved in the progression of PCa from androgen dependence to independence (Murillo *et al.* 2001, Kikuchi *et al.* 2003).

The phosphatidylinositol 3-kinase (PI3K)/AKT cell signaling pathway is an important regulator of growth and survival in many cell types including prostate. Although the relationship between AR and PI3K/AKT remains controversial, a variety of mechanisms have been suggested to account for how AKT influences AR signaling pathways. These include the followings: 1) AKT directly interacts with AR and then suppresses AR activity (Lin *et al.* 2001, Yang *et al.* 2003*a*,*b*); 2) AKT directly interacts with AR and then enhances AR activity in a ligand-independent manner

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(Wen *et al.* 2000); 3) AKT indirectly enhances AR transactivation via the inhibition of GSK3B, the downstream substrate of PI3K/AKT, protein kinase A, and mitogen-activated protein kinase; GSK3B was found to suppress directly AR transactivation (Wang *et al.* 2004); and 4) AKT indirectly enhances AR transactivation by inhibiting GSK3B to positively regulate β-catenin (Sharma *et al.* 2002). β-catenin has been reported as a ligand-dependent AR co-activator (Truica *et al.* 2000). How AKT influences AR may depend on cell physiological conditions. AKT can activate NFKB pathway via phosphorylation and activation of molecules in the NFKB pathway (Ozes *et al.* 1999, Romashkova & Makarov 1999).

It has been well known that the NFKB pathway plays an important role in the control of cell growth, differentiation, apoptosis, inflammation, stress response, and many other physiological processes in cellular signaling. The NFKB signaling pathway is also involved in the development and progression of PCa. NFKB is overexpressed in prostatic intraepithelial neoplasia and prostate adenocarcinoma (Sweeney *et al.* 2004). Constitutive activation of NFKB has been found in androgen-independent PCa cells, whereas less activity of NFKB has been observed in androgen-dependent PCa cells (Suh *et al.* 2002, Zerbini *et al.* 2003). Like AKT and AR, the relationship between NFKB and AR activation remains controversial. Palvimo *et al.* (1996) reported that elevated expression of NFKB p65 repressed AR-mediated transactivation in a dose-dependent manner, whereas NFKB p50 did not influence AR transactivation. However, other investigators have shown that IL4-induced NFKB is required for AR activation (Lee *et al.* 2005), and that there are NFKB binding sites in the promoter of AR (Zhang *et al.* 2004), suggesting that the activation of NFKB could enhance AR transactivation.

To clarify these issues, we studied the direct effect of DHEA on primary cultured rat prostatic epithelial cells in the presence/absence of Casodex and *Ar* siRNA, both of which are recognized blockers of AR. We have analyzed the effect of DHEA on epithelial cell proliferation and the activity of downstream gene products playing a prominent role in prostatic epithelial cells. Our results show that DHEA stimulated PI3K/AKT activity and finally activates the pro-survival transcription factor NFKB in prostatic epithelial cells.

Materials and Methods

Rat ventral prostate epithelial cell culture

Primary culture of rat prostatic epithelial cells was carried out as described previously: prostates were taken from 6-week-old animals (Taketa *et al.* 1990). Cells were cultured in 2 ml of a medium consisting of RPMI-1640 (Gibco) supplemented with 10% fetal bovine serum, glutamine 2 mmol/l, penicillin 100 kU/l, streptomycin 100 mg/l, epidermal growth factor 10 μg/l, cholera toxin 10 μg/l, and transferrin 5 mg/l. Cultures were incubated in a humidified atmosphere of 5% carbon dioxide and 95% air at 37 °C. Upon reaching monolayer confluence, the cells were split by treatment with 0·25% trypsin solution to give a final concentration of 5×10^4 cells/ml with PBS before starvation in 0·1% BSA (fraction V, Sigma), phenol red-free and serum-free RPMI-1640 for further 24 h, and the following tests were carried out.

In this study, DHEA, dihydrotestosterone (DHT), and Casodex (bicalutamide) were all purchased from Sigma–Aldrich.

^{3}H-thymidine incorporation

To assess cell proliferation in prostatic epithelial cells, we measured ^{3}H-thymidine incorporation into newly synthesized DNA. At the end of the culture period, 2·5 μCi/well of ^{3}H-thymidine (25 Ci/mmol; Amersham) was added to the culture medium for an additional 3 h. Cells were released by trypsin and collected onto glass fiber filters. Incorporation of ^{3}H-thymidine was measured by liquid scintillation counting. Five replicates per condition were assayed, and data expressed as mean ± S.E.M. from three separate experiments were presented.

Ar siRNA transfection

Prostatic epithelial cells were transfected with siRNA targeted for AR (Santa Cruz Biotechnology, Santa Cruz, CA, USA). We used a pool of four target-specific, 20–25 nucleotide-long siRNAs designed to knock down AR gene expression. Sense strand (A): CUG AGU AUU CCU CUU UCA A; sense strand (B): CAG UCC CAA UUG UGU CAA A; sense strand (C): CCA GAA GAU GAC UGU AUC A; sense strand (D): GAC UGU AUC ACA CAU UGA A. An siRNA consisting of a scrambled sequence was similarly transfected as control. siRNA was introduced to cells using Lipofectamine 2000 (Invitrogen Corporation), according to the procedure recommended by the manufacturer. One day before transfection, the cells were plated in 500 μl growth medium without antibiotics such that they were 30–50% confluent at the time of transfection. The transfected cells were cultured in DMEM containing 10% FCS for 72 h after transfection.

To determine transfection efficiency, we transfected prostatic epithelial cells with FITC-conjugated control siRNA (Santa Cruz Biotechnology) in two separate experiments. Transfection efficiency was 57·0 ± 3·1% (range of 46·8–60·4%). To demonstrate the specific inhibitory effect of *Ar* siRNA on AR expression, we evaluated *Ar* mRNA and protein expression in transfected prostatic epithelial cells by real-time PCR and western blot respectively.

Real-time PCR

At the end of the culture period, total RNA was extracted from prostatic epithelial cells using the Qiagen RNeasy Mini kit (Qiagen Inc). The recovered RNA was further processed using 1st Strand cDNA synthesis kit for RT-PCR

(avian myeloblastosis virus (AMV); Roche Diagnostics Corp.) to produce cDNA according to the manufacturer's instruction. Real-time quantitative PCR was carried out using StepOne real-time PCR system (Applied Biosystems, Foster City, CA, USA) in a final volume of 25 μl containing 1 μl cDNA, 12·5 μl 2× SYBR Green Master Mix (Applied Biosystems), 0·1 μM primers (Applied Biosystems) in DNase-free water. The PCR conditions were: 50 °C for 2 min followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The size of the real-time PCR products was verified by electrophoresis on 4% agarose gels. In addition, dissociation curve analysis was also performed after each PCR to ensure that a single product and no primer–dimers were present. Relative mRNA expression level of *Ar* and *Psa* (listed as *Klkb1* in the MGI Database) was calculated using the $\Delta\Delta C_t$ method, where the calibrator is the β-actin gene.

Western blot

Whole cell lysates were solubilized with 1% SDS sample buffer and electrophoresed on a 4–15% SDS-PAGE gel (Bio-Rad). Proteins were transferred onto a nitrocellulose membrane and were probed with the following primary antibodies: rabbit polyclonal antibodies against β-actin and AKT (Santa Cruz) and goat polyclonal antibody against PSA (Santa Cruz). The blots were developed using a HRP-conjugated polyclonal goat-anti rabbit IgG or a donkey anti-goat IgG antibody and enhanced chemiluminescence system (Amersham). The protein size was confirmed by molecular weight standards (Invitrogen). The intensity of the bands on western blots was analyzed by Image J (software from NCBI; http://rsb.into.nih.gov/ij/). Results were expressed as percentage control siRNA-transfected cells and were obtained from three separate experiments.

Electrophoretic mobility shift assay

Nuclear protein extract was prepared from cultured prostatic epithelial cells (Wu *et al*. 2001). NFKB binding activity was studied by using double-stranded oligonucleotides (5′-AGT TGA GGG GAC TTT CCC AGG C-3′, corresponding to the consensus NFKB binding site, Promega). The oligonucleotide probe was prepared by phosphorylation with T4 polynucleotide kinase (Promega Corporation) in the presence of [γ-^{32}P] ATP (Amersham Biosciences), followed by inactivation of the kinase by adding 1 μl of 0·5 M EDTA. Nuclear proteins (10 μg) were preincubated for 10 min in NFKB binding buffer (Promega). Radioactively labeled oligonucleotide was added, and the mixture was incubated for 30 min at room temperature. The complexes were then subjected to 6% nondenaturing acrylamide gel, electrophoresed, and analyzed by autoradiography. To assess the specificity of the *Nfkb* DNA binding, competition experiments were performed by using excess (10×) of unlabeled *Nfkb* oligonucleotides and nonspecific competitor DNA sequence (SP1).

PI3K assay

PI3K activities were determined by an *in vitro* kinase assay (SuperArray Bioscience Corporation, Frederick, MD, USA), according to manufacturer's instructions. Cells are seeded into 96-well plates and incubated with DHEA, DHT and/or Casodex, and/or *Ar* siRNA for 24 h. Cells were then fixed with 4% formaldehyde for 20 min at room temperature to preserve phosphorylation. The relative extent of target protein phosphorylation is determined by normalizing absorbance reading of the phospho-protein specific antibody to the pan-protein specific antibody for the same experimental condition. Experiments were performed more than three times, and data were expressed as percentage of control.

Nfkb p65 transcription factor assay

Nfkb p65 transcription factor activity was determined by an ELISA (Cayman Chemical, Ann Arbor, MN, USA), according to the manufacturer's instructions. Nuclei were extracted from prostatic epithelial cells treated for 24 h with 100 nM DHEA, 100 nM DHT, and/or the following specific protein kinase inhibitors: Wortmannin (PI3K inhibitor); Akti-1/2 (AKT inhibitor). All these inhibitors (EMD Chemicals, Gibbstown, NJ, USA) have previously been shown to be specific inhibitors (Bain *et al*. 2007). Data are expressed as absorbance at 450 nm/μg protein. The results are expressed as the mean ± S.E.M. of three separate experiments.

Animal treatment

All animals used in these studies were maintained in compliance with the Animal Experiments Guidelines and Animal Care of Chinese Academy of Sciences. Sprague–Dawley male rats of age 6 weeks were housed in groups of two per individual microisolator cage under controlled temperature (21–22 °C), humidity (50%), and light conditions (12 h light:12 h darkness cycle; lights on at 0700 h).

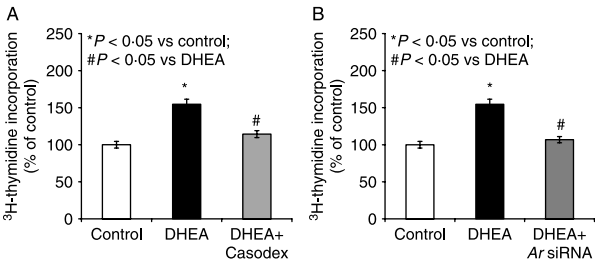

Figure 1 Effect of DHEA on rat prostatic epithelial cell proliferation. Rat prostatic epithelial cells were treated with DHEA in the presence of Casodex (A) or *Ar* siRNA (B) for 24 h. At the end of the culture period, 2·5 μCi/well of ^{3}H-thymidine was added to the culture medium for an additional 3 h. Cells were released by trypsin and collected onto glass fiber filters. Incorporation of ^{3}H-thymidine was measured by liquid scintillation counting. Data were expressed by percentage of control (mean ± S.E.M.) and were obtained from three separate experiments.

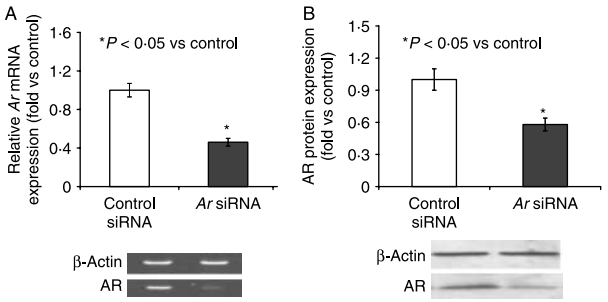

Figure 2 Effect of siRNA on *Ar* gene knocking down. (A) *Ar* mRNA expression was detected by real-time PCR after transfected with *Ar* siRNA. A representative blot from three independent experiments is presented. Results are expressed as percentage control siRNA-transfected cells and were obtained from three separate experiments. (B) Transfected chondrocytes were harvested, lysed, electrophoresed, and immunoblotted for AR and the loading control, β-actin. A representative blot from three independent experiments is presented. The intensity of the bands on western blots was analyzed by Image J (software from NCBI). Results are expressed as percentage control siRNA-transfected cells (mean ± S.E.M.) and were obtained from three separate experiments.

Animals were fed a commercially available chow, and water was available *ad libitum*. Castration was performed via a scrotal incision under ether anesthesia before starting the experiment. Both the testes and epididymis were removed. After castration, the rats were maintained under standard laboratory conditions for 7 days. Then the animals were randomized according to their body weights and were assigned to three groups of 9–10 animals each as follows: 1) gonadectomized (GDX) control; 2) GDX + DHEA (3 mg/rat diet) and 3) GDX + DHT (0·1 mg/rat). DHEA or DHT suspended in 5% ethanol–0·4% methylcellulose was administered s.c. once a day at 0800 h, while GDX control group received vehicle alone during the same time period. The volume of an individual gastric feeding was 1·0 ml/100 g body weight every day for 30 days. Prostates of ten rats in each group were obtained for investigation 24 h after the last administration. After weighing, RNA and protein were extracted from ventral prostate, and *Psa* mRNA and protein levels were determined by quantitative RT-PCR and western blot. Experiments were performed twice, and data were expressed as fold of control.

Statistical analysis

Statistical differences were determined by a Student's *t*-test. A P value $<0{\cdot}05$ was considered significant. Values are the mean ± S.E.M.

Results

Effect of DHEA on prostatic epithelial cell proliferation

Prostatic epithelial cell cultures were treated with 100 nM DHEA for up to 24 h. When compared with control, DHEA exhibited increased cell proliferation (assessed by total ^{3}H-thymidine incorporation, 154·6 ± 6·8% of control, $P<0{\cdot}05$, Fig. 1A), while addition of Casodex neutralized this stimulating effect induced by DHEA (DHEA + Casodex versus DHEA: 114·3 ± 4·7 vs 154·6 ± 6·8% of control, $P<0{\cdot}05$; Fig. 1A). Blocking AR by *Ar* siRNA inhibited DHEA-induced proliferation of prostatic epithelial cell similar to that observed using pharmacological blockade by Casodex (DHEA + *Ar* siRNA versus DHEA: 106·8 ± 4·2 vs 154·6 ± 6·8% of control, $P<0{\cdot}05$, Fig. 1B). Diminished *Ar* mRNA (0·46 ± 0·05-fold compared with control, $P<0{\cdot}01$, Fig. 2A) and protein levels (0·58 ± 0·06-fold compared with control, $P<0{\cdot}01$, Fig. 2B) represented by real-time PCR and western blot confirm effectiveness of *Ar* siRNA.

Effect of DHEA on Psa mRNA and protein expression on prostatic epithelial cell

PSA is a clinically important marker used to monitor diagnosis, treatment response, prognosis, and progression in patients with PCa (Zhang *et al*. 2004). To investigate whether androgenic pathways involved in DHEA-mediated effects in prostate cells, *Psa* gene and protein expression were evaluated in the absence and presence of AR antagonists, Casodex or

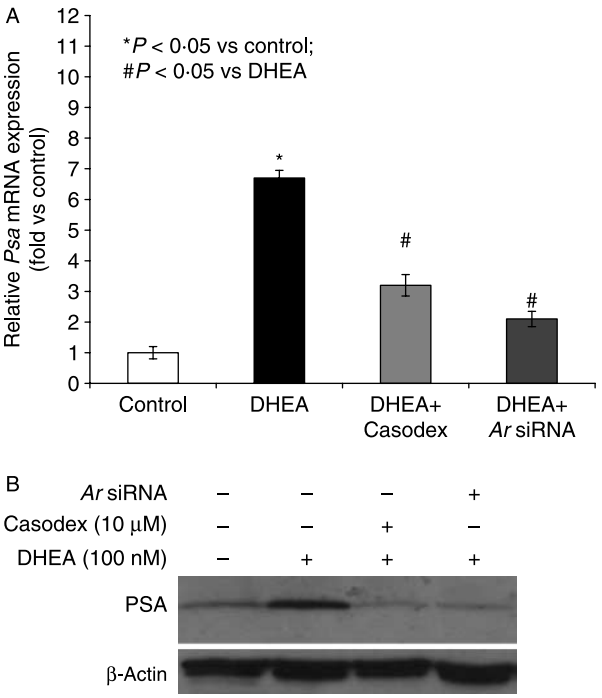

Figure 3 Effects of DHEA on *Psa* mRNA and protein expression. (A) *Psa* mRNA expression was detected by real-time PCR. The housekeeping gene β-actin was used as normalization control. Results were expressed as fold change compared to control epithelial cells (mean ± S.E.M). (B) At the end of the culture period, cells were harvested, lysed, electrophoresed, and immunoblotted for PSA and the loading control, β-actin. A representative blot from three independent experiments is presented.

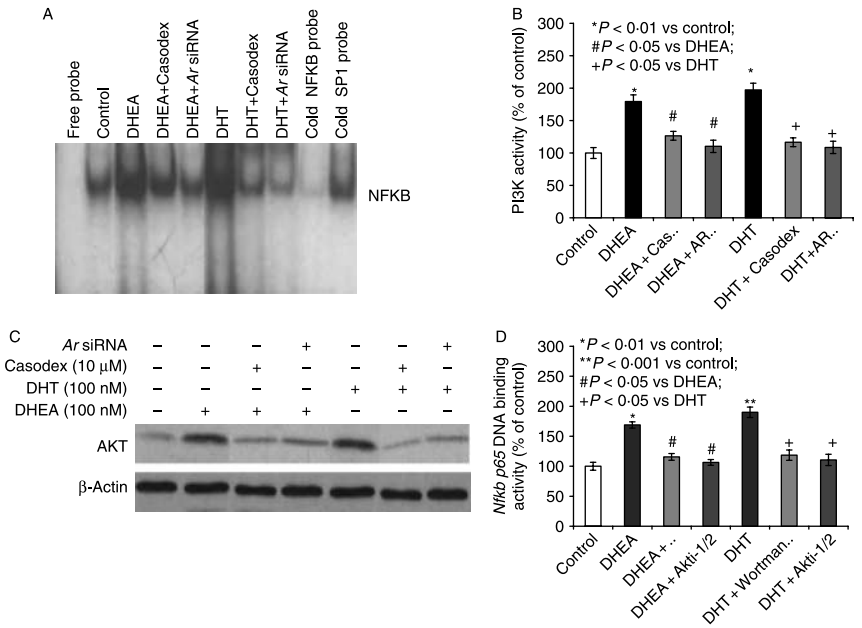

Figure 4 Effects of DHEA and DHT on *Nfkb* DNA binding activity and PI3K/AKT activity with/without AR blockers. (A) Prostatic epithelial cells were cultured without or with Casodex at the time points indicated. A labeled oligonucleotide of NFKB consensus element was incubated with the prostatic cells nuclear extract. DNA binding was analyzed by EMSA. The arrow indicates the NFKB–DNA complex. Representative results of three experiments are depicted. For specificity, *Nfkb* DNA binding was competed out with a NFKB cold probe (CP1) and with the SP1 cold probe (CP2). (B) PI3K activity was determined by an *in vitro* kinase assay. (C) AKT expression was analyzed by western blot. (D) *Nfkb p65* DNA binding activity was determined by an ELISA, according to the manufacturer's instructions. Data were expressed by mean ± S.E.M. and were obtained from three separate experiments.

Ar siRNA. DHEA significantly increased *Psa* mRNA 6·7 ± 0·25-fold of control, an effect blocked by Casodex or *Ar* siRNA (3·2 ± 0·35 and 2·1 ± 0·21-fold of control respectively; Fig. 3A, $P<0·05$). PSA protein expression was stimulated 5·3 ± 0·17-fold of control by DHEA, whereas co-treatment with Casodex or *Ar* siRNA decreased DHEA-stimulated PSA levels to 2·8 ± 0·24 and 2·1 ± 0·16-fold of control respectively (Fig. 3B).

Effect of DHEA on Nfkb DNA binding activity

To determine whether the DHEA-mediated proliferation led to an increase of NFKB activation, we studied the binding of NFKB to DNA by performing electrophoretic mobility shift assay. Epithelial cells isolated from rat prostate were cultured up to 24 h with or without DHEA in the presence or absence of Casodex or *Ar* siRNA, and nuclear extracts were then prepared. Labeled oligonucleotides containing a NFKB consensus sequence were incubated with prostatic cells nuclear extracts, leading to the formation of a protein–DNA complex. DHEA stimulated formation of the NFKB–DNA complex at 24 h of culture, with this effect being neutralized by addition of Casodex or *Ar* siRNA. To confirm specificity, *Nfkb* DNA binding was competed out with a NFKB cold probe but not with the SP1 cold probe (Fig. 4A). Similarly, formation of the NFKB–DNA complex stimulated by DHT was also neutralized by addition of Casodex or *Ar* siRNA.

Effect of DHEA on PI3K/AKT signaling pathway

Previous studies identified a PI3K/c-AKT/Pak1/NFKB cell survival pathway in DU-145 PCa and Madin–Darby canine kidney epithelial cells (Cleutjens *et al.* 1996). To determine if intracellular signaling pathway(s) PI3K/AKT are involved in prostatic epithelial cell physiology, we first determined PI3K/AKT activity in prostatic epithelial cells treated by DHEA or DHT in the presence or absence of Casodex or *Ar* siRNA. DHEA stimulated PI3K/AKT dramatically (179·4 ± 10·3% of control, $P<0·05$), with this stimulation being neutralized by the addition of Casodex or *Ar* siRNA (DHEA + Casodex versus DHEA: 126·6 ± 6·8 vs 179·4 ± 10·3% of control; DHEA + *Ar* siRNA versus DHEA: 110·3 ± 9·5 vs 179·4 ± 10·3% of control; $P<0·05$, Fig. 4B and C). Similarly, stimulation of PI3K/AKT activity caused by DHT (197·4 ± 8·7% of control, $P<0·01$) was neutralized by the addition of Casodex or *Ar* siRNA (DHT + Casodex versus DHT: 116·6 ± 6·8 vs 197·4 ± 8·7% of control; DHT + *Ar*

siRNA versus DHT: 108·7±9·5 vs 197·4±8·7% of control; $P<0·05$, Fig. 4B and C). To confirm if PI3K/AKT mediates the effects of DHEA on NFKB p65 activity, we cultured epithelial cells in the presence of DHEA, with or without specific PI3K/AKT inhibitor. Both DHEA and DHT stimulated NFKB p65 DNA binding activity dramatically (DHEA: 168·8±5·2% of control; $P<0·01$; DHT: 189·9±8·7% of control, $P<0·001$). The addition of 10 μM wortmannin (a PI3K inhibitor) or 10 μM Akti-1/2 (AKT inhibitor) to the culture medium of DHEA-treated prostatic epithelial cells significantly reversed the stimulatory effects of DHEA on *Nfkb p65* DNA binding activity (DHEA + Wortmannin versus DHEA: 115·6±5·6 vs 168·8±5·2% of control; $P<0·05$; DHEA + Akti-1/2 versus DHEA: 106·4 ±4·8 vs 168·8±5·2% of control, $P<0·05$; Fig. 4D). Similar to this finding, Wortmannin or Akti-1/2 also blocked NFKB activity induced by 100 nM DHT (DHT + Wortmannin versus DHT: 118·5±8·6 vs 189·9±8·7% of control; $P<0·01$; DHT + Akti-1/2 versus DHT: 110·6±9·4 vs 189·9±8·7% of control, $P<0·05$; Fig. 4D).

Effect of DHEA and DHT on PSA mRNA and protein expression of castrated rats

Male rats given DHEA daily for 30 days did not show any statistical difference in body weight (data not shown). Mean weight of ventral prostate weight (normalized to mg/100 g animal body weight) in GDX rats was significantly increased by chronic exposure to either DHEA or DHT (DHEA versus control: 23·9±7·5 vs 9·6±3·8, $P<0·05$; DHT versus control: 31·4±6·7 vs 9·6±3·8, $P<0·01$, Fig. 5A).

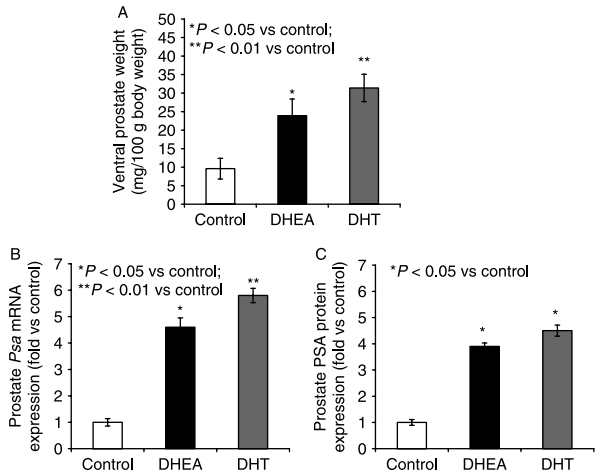

Figure 5 Effect of DHEA and DHT on prostate weight, prostatic *Psa* mRNA and protein expression in castrated rats. Prostates of ten rats in each group were obtained for investigation 24 h after the last administration. Ventral prostate weight (A), RNA and protein were extracted from ventral prostate, and *Psa* mRNA (B) and protein (C) were performed by quantitative RT-PCR and western blot. Data were expressed by mean±S.E.M. and were obtained from two separate experiments.

Both DHEA and DHT significantly increased *Psa* mRNA expression in prostate isolated from male rats, as evaluated by quantitative RT-PCR (4·6±0·35- and 5·8±0·27-fold versus control respectively, $P<0·05$ DHEA versus control and $P<0·01$ DHT versus control, Fig. 5B); furthermore, DHEA and DHT also induced a marked increase of PSA protein expression (3·9±0·21-fold of control and 4·5±0·13-fold of control respectively, $P<0·05$; Fig. 5C).

Discussion

In the United States, DHEA is widely used as an over-the-counter dietary supplement on the basis of its purported, yet controversial, anti-aging benefits to improve body composition, endocrine-metabolic balance, immune and psychological functions, and quality of life (Svec & Porter 1998, Alesci *et al.* 2005). Because DHEA is a precursor of more potent androgens and estrogens, there is some concern that DHEA supplementation may promote PCa growth or deleterious functions in men with preexisting PCa (Acacio *et al.* 2004). This study aimed to evaluate the direct effects of DHEA on prostatic epithelial cells.

We found evidence to suggest that DHEA stimulated prostatic epithelial cells proliferation through AR. DHEA-induced proliferation of the prostatic epithelial cells was blunted by the addition of Casodex, an antagonist of AR or siRNA, indicating that the effect of DHEA on epithelial cells was by acting through AR. It was also reported that the AR antagonist, Casodex, inhibited DHEA-induced proliferation of LNCaP cells (Arnold *et al.* 2007). Interactions among androgens, estrogens, and their receptors contribute to normal prostate development and function (Prins *et al.* 1998, Risbridger *et al.* 2003, Ho 2004, Soronen *et al.* 2004), and to dysregulation of prostate growth, potentially promoting hormone-independent PCa (Arnold & Isaacs 2002, Heinlein & Chang 2004). In castrated rats, our result demonstrated that DHEA treatment had no significant effect in body weight. Consistent to our finding, previous studies have shown that administration of DHEA at dietary concentrations of up to ten times this level has no effect on body weight gain in Sprague–Dawley rats (McCormick *et al.* 1996). Previous *in vivo* study demonstrated that DHEA replacement had no significant effect on serum PSA level in healthy aged men (Jedrzejuk *et al.* 2003); DHEA-S serum levels were negatively correlated with patient age, sexual function score, total score, and PSA (Ponholzer *et al.* 2002). In our study, however, similar to the stimulation effect on prostate induced by DHT, a positive control, DHEA treatment for 30 days increased prostate weight, as well as *Psa* mRNA and protein expression level in prostate. *Psa* is a well-known AR-regulated gene in the human prostate gland, and is expressed principally by both normal prostate epithelium and hormone-dependent PCa cells. PSA is generally considered to be the most sensitive biochemical marker available for monitoring the presence of

prostatic disease, particularly PCa, and response to therapy (Heinlein & Chang 2004). The primary regulator of PSA expression is AR, which induces PSA expression through three ARE-containing enhancer elements located in the proximal 6 kb of the PSA promoter (Cleutjens *et al*. 1997). Our finding demonstrated that DHEA stimulated PSA on epithelial cells compared with untreated cells, while co-treatment with Casodex or siRNA neutralized such effect. In LNCaP cells, DHEA-stimulated PSA gene and protein expression were inhibited by Casodex. Blocking AR by Casodex or by siRNA inhibited the effects of androgenic hormones.

Previous studies identified a PI3K/c-AKT/Pak1/NFKB cell survival pathway in DU-145 PCa and Madin–Darby canine kidney epithelial cells (Ross *et al*. 2003). In clinical studies of PCa specimens, overexpression of NFKB/p65 protein was shown to be an independent predictor of poor prognosis in PCa patients (Fradet *et al*. 2004). In prostatectomy specimens of PCa with relapsed tumor, NFKB was found to be concentrated in the nuclear fraction (Ross *et al*. 2003). Therefore, effective inhibition of NFKB could be critical in providing a targeted pathway for PCa prevention. It was also observed that treatment of PC3 with delphinidin (fruit- and vegetable-derived chemopreventive agents) led to a dose-dependent decrease in the DNA binding potential of NFKB, thereby making it transcriptionally incompetent to drive the expression of target genes. In our study, DHEA-induced *Nfkb* DNA binding activity on prostatic epithelial cell proliferation and PSA expression was neutralized by addition of *Ar* siRNA. Using specific chemical inhibitors, we also demonstrate involvement of PI3K/AKT signaling in NFKB-mediated regulation of DHEA activities in prostatic epithelial cells. The PI3K/AKT cell signaling pathway is an important regulator of growth and survival in many cell types including prostate (Bellacosa *et al*. 1991, Nakatani *et al*. 1999). AKT can be activated by various growth factors by activating PI3K and subsequently phosphorylating AKT at Thr308 or Ser473 (Burgering & Coffer 1995, Franke *et al*. 1995, Alessi *et al*. 1996, Okano *et al*. 2000). Our data are concordant with the fact that constitutive activation of AKT has been reported in PC cells, which in turn stimulates the transactivation potential of the RelA/p65 subunit of NFKB through utilization of the IκB kinase and activation of the mitogen-activated protein kinase p38 (Madrid *et al*. 2001). Reports on the role of NFKB in AR signaling are conflict. Some showed NFKB negatively regulates AR function (Palvimo *et al*. 1996), and others showed NFKB enhances AR-mediated PSA expression (Chen & Sawyers 2002). Our study clarified that under normal condition, NFKB activation facilitates DHEA-induced prostatic epithelial cell proliferation and PSA expression through PI3K/AKT pathway. In summary, our study demonstrates that DHEA stimulates prostatic epithelial cells proliferation and PSA expression by activating, at least in part, NFKB via the PI3K/AKT pathway.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References

Acacio BD, Stanczyk FZ, Mullin P, Saadat P, Jafarian N & Sokol RZ 2004 Pharmacokinetics of dehydroepiandrosterone and its metabolites after long-term daily oral administration to healthy young men. *Fertility and Sterility* **81** 595–604.

Alesci S, Manoli I & Blackman MR 2005 Dehydrodepiandrosterone (DHEA). In *Encyclopedia of Dietary Supplements*, 1 edn, pp 167–176. Ed. P Coates. New York, NY: Marcel Dekker, Inc.

Alessi DR, Andjelkovic M, Caudwell B, Cron P, Morrice N, Cohen P & Hemmings BA 1996 Mechanism of activation of protein kinase B by insulin and IGF-1. *EMBO Journal* **15** 6541–6551.

Arnold JT 2009 DHEA metabolism in prostate: for better or worse. *Molecular and Cellular Endocrinology* **301** 83–88.

Arnold JT & Isaacs JT 2002 Mechanisms involved in the progression of androgen-independent prostate cancers: it is not only the cancer cell's fault. *Endocrine-Related Cancer* **9** 61–73.

Arnold JT, Le H, McFann KK & Blackman MR 2005 Comparative effects of DHEA vs. testosterone, dihydrotestosterone, and estradiol on proliferation and gene expression in human LNCaP prostate cancer cells. *American Journal of Physiology. Endocrinology and Metabolism* **288** E573–E584.

Arnold JT, Liu X, Allen JD, Le H, McFann KK & Blackman MR 2007 Androgen receptor or estrogen receptor-beta blockade alters DHEA-, DHT-, and E(2)-induced proliferation and PSA production in human prostate cancer cells. *Prostate* **67** 1152–1162.

Bain J, Plater L, Elliott M, Shpiro N, Hastie CJ, McLauchlan H, Klevernic I, Arthur SC, Alessi DR & Cohen P 2007 The selectivity of protein kinase inhibitors: a further update. *Biochemical Journal* **408** 297–315.

Bellacosa A, Testa JR, Staal SP & Tsichlis PN 1991 A retroviral oncogene, akt, encoding a serine–threonine kinase containing an SH2-like region. *Science* **254** 274–277.

Burgering BM & Coffer PJ 1995 Protein kinase B (c-Akt) in phosphatidyl-inositol- 3-OH kinase signal transduction. *Nature* **376** 599–602.

Chen CD & Sawyers CL 2002 NF-kappa B activates prostate-specific antigen expression and is upregulated in androgen-independent prostate cancer. *Molecular and Cellular Biology* **22** 2862–2870.

Chen F, Knecht K, Birzin E, Fisher J, Wilkinson H, Mojena M, Moreno CT, Schmidt A, Harada S, Freedman LP *et al*. 2005 Direct agonist/antagonist functions of dehydroepiandrosterone. *Endocrinology* **146** 4568–4576.

Cleutjens KBJM, van Eekelen CCEM, Van der Korput HAGM, Brinkman AO & Trapman J 1996 Two androgen response regions cooperate in steroid hormone regulated activity of the prostate specific antigen promoter. *Journal of Biological Chemistry* **271** 6379–6388.

Cleutjens KBJM, Van der Korput HAGM, van Eekelen CCEM, van Rooij HCJ, Faber PW & Trapman J 1997 An androgen response element in a far upstream enhancer region is essential for high, androgen-regulated activity of the prostate-specific antigen promoter. *Molecular Endocrinology* **11** 148–161.

Fradet V, Lessard L, Begin LR, Karakiewicz P, Masson AM & Saad F 2004 Nuclear factor-κB nuclear localization is predictive of biochemical recurrence in patients with positive margin prostate cancer. *Clinical Cancer Research* **10** 8460–8464.

Franke TF, Yang SI, Chan TO, Datta K, Kazlauskas A, Morrison DK, Kaplan DR & Tsichlis PN 1995 The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF activated phosphatidylinositol 3-kinase. *Cell* **81** 727–736.

Heinlein CA & Chang C 2004 Androgen receptor in prostate cancer. *Endocrine Reviews* **25** 276–308.

Ho SM 2004 Estrogens and anti-estrogens: key mediators of prostate carcinogenesis and new therapeutic candidates. *Journal of Cellular Biochemistry* **91** 491–503.

Jedrzejuk D, Medras M, Milewicz A & Demissie M 2003 Dehydroepiandrosterone replacement in healthy men with age-related decline of DHEA-S:effects on fat distribution, insulin sensitivity and lipid metabolism. *Aging Male* **6** 151–156.

Kikuchi E, Horiguchi Y, Nakashima J, Kuroda K, Oya M, Ohigashi T, Takahashi N, Shima Y, Umezawa K & Murai M 2003 Suppression of hormone-refractory prostate cancer by a novel nuclear factor κB inhibitor in nude mice. *Cancer Research* **63** 107–110.

Klein H, Molwitz T & Bartsch W 1989 Steroid sulfate sulfatase in human benign prostatic hyperplasia: characterization and quantification of the enzyme in epithelium and stroma. *Journal of Steroid Biochemistry* **33** 195–200.

Labrie F, Bélanger A, Luu-The V, Labrie C, Simard J, Cusan L, Gomez JL & Candas B 1998 DHEA and the intracrine formation of androgens and estrogens in peripheral target tissues: its role during aging. *Steroids* **63** 322–328.

Labrie F, Luu-The V, Labrie C & Simard J 2001 DHEA and its transformation into androgens and estrogens in peripheral target tissues: intracrinology. *Frontiers in Neuroendocrinology* **22** 185–212.

Lee SO, Lou W, Nadiminty N, Lin X & Gao AC 2005 Requirement for NF-(κ)B in interleukin-4-induced androgen receptor activation in prostate cancer cells. *Prostate* **64** 160–167.

Lin HK, Yeh S, Kang HY & Chang C 2001 Akt suppresses androgen-induced apoptosis by phosphorylating and inhibiting androgen receptor. *PNAS* **98** 7200–7205.

Madrid LV, Mayo MW, Reuther JY & Baldwin AS Jr 2001 Akt stimulates the transactivation potential of the RelA/p65 subunit of NF-κB through utilization of the IκB kinase and activation of the mitogen-activated protein kinase p38. *Journal of Biological Chemistry* **276** 18934–18940.

McCormick DL, Rao KVN, Johnson WD, BowmanGram TA, Steele VE, Lubet RA & Kelloff GJ 1996 Exceptional chemopreventive activity of low-dose dehydroepiandrosterone in the rat mammary gland. *Cancer Research* **56** 1724–1726.

Murillo H, Huang H, Schmidt LJ, Smith DI & Tindall DJ 2001 Role of PI3K signaling in survival and progression of LNCaP prostate cancer cells to the androgen refractory state. *Endocrinology* **142** 4795–4805.

Nakatani K, Sakaue H, Thompson DA, Weigel RJ & Roth RA 1999 Identification of a human Akt3 (protein kinase B gamma) which contains the regulatory serine phosphorylation site. *Biochemical and Biophysical Research Communications* **257** 906–910.

Okano J, Gaslightwala I, Birnbaum MJ, Rustgi AK & Nakagawa H 2000 Akt/protein kinase B isoforms are differentially regulated by epidermal growth factor stimulation. *Journal of Biological Chemistry* **275** 30934–30942.

Ozes ON, Mayo LD, Gustin JA, Pfeffer SR, Pfeffer LM & Donner DB 1999 NF-kappaB activation by tumour necrosis factor requires the Akt serine–threonine kinase. *Nature* **401** 82–85.

Palvimo JJ, Reinikainen P, Ikonen T, Kallio PJ, Moilanen A & Janne OA 1996 Mutual transcriptional interference between RelA and androgen receptor. *Journal of Biological Chemistry* **271** 24151–24156.

Ponholzer A, Plas E, Schatzl G, Jungwirth A & Madersbacher S 2002 Association of DHEA-S and estradiol serum levels to symptoms of aging men. *Aging Male* **5** 233–238.

Prins GS, Marmer M, Woodham C, Chang W, Kuiper G, Gustafsson JA & Birch L 1998 Estrogen receptor-beta messenger ribonucleic acid ontogeny in the prostate of normal and neonatally estrogenized rats. *Endocrinology* **139** 874–883.

Risbridger GP, Bianco JJ, Ellem SJ & McPherson SJ 2003 Oestrogens and prostate cancer. *Endocrine-Related Cancer* **10** 187–191.

Romashkova JA & Makarov SS 1999 NF-kappaB is a target of AKT in anti-apoptotic PDGF signalling. *Nature* **401** 86–90.

Ross JS, Jennings TA, Nazeer T, Sheehan CE, Fisher HA, Kauffman RA, Anwar S & Kallakury BV 2003 Prognostic factors in prostate cancer. *American Journal of Clinical Pathology* **120** 85–100.

Sharma M, Chuang WW & Sun Z 2002 Phosphatidylinositol 3-kinase/Akt stimulates androgen pathway through GSK3β inhibition and nuclear β-catenin accumulation. *Journal of Biological Chemistry* **277** 30935–30941.

Soronen P, Laiti M, Torn S, Harkonen P, Patrikainen L, Li Y, Pulkka A, Kurkela R, Herrala A, Kaija H *et al.* 2004 Sex steroid hormone metabolism and prostate cancer. *Journal of Steroid Biochemistry and Molecular Biology* **92** 281–286.

Suh J, Payvandi F, Edelstein LC, Amenta PS, Zong WX, Gélinas C & Rabson AB 2002 Mechanisms of constitutive NF-κB activation in human prostate cancer cells. *Prostate* **52** 183–200.

Svec F & Porter JR 1998 The actions of exogenous dehydroepiandrosterone in experimental animals and humans. *Proceedings of the Society for Experimental Biology and Medicine* **218** 174–191.

Sweeney C, Li L, Shanmugam R, Bhat-Nakshatri P, Jayaprakasan V, Baldridge LA, Gardner T, Smith M, Nakshatri H & Cheng L 2004 Nuclear factor-κB is constitutively activated in prostate cancer *in vitro* and is overexpressed in prostatic intraepithelial neoplasia and adenocarcinoma of the prostate. *Clinical Cancer Research* **10** 5501–5507.

Taketa S, Nishi N, Takasuga H, Okntani T, Takenaka I & Wada F 1990 Difference in growth requirements between epithelial and stromal cells derived from rat ventral prostate in serum-free primary culture. *Prostate* **17** 207–218.

Truica CI, Byers S & Gelmann EP 2000 Beta-catenin affects androgen receptor transcriptional activity and ligand specificity. *Cancer Research* **60** 4709–4713.

Wang L, Lin HK, Hu YC, Xie S, Yang L & Chang C 2004 Suppression of androgen receptor-mediated transactivation and cell growth by glycogen synthase kinase 3β in prostate cells. *Journal of Biological Chemistry* **279** 32444–32452.

Wen Y, Hu MC, Makino K, Spohn B, Bartholomeusz G, Yan DH & Hung MC 2000 HER-2/neu promotes androgen-independent survival and growth of prostate cancer cells through the Akt pathway. *Cancer Research* **60** 6841–6845.

Widstrom RL & Dillon JS 2004 Is there a receptor for dehydroepiandrosterone or dehydroepiandrosterone sulfate? *Seminars in Reproductive Medicine* **22** 289–298.

Wu SF, Sun HZ, Qi XD & Tu ZH 2001 Effect of episteride on the expression of IGF-1 and TGF-beta receptors in androgen-induced castrated rat prostate. *Experimental Biology and Medicine* **226** 954–960.

Yang L, Lin HK, Altuwaijri S, Xie S, Wang L & Chang C 2003*a* APPL suppresses androgen receptor transactivation via potentiating Akt activity. *Journal of Biological Chemistry* **278** 16820–16827.

Yang L, Wang L, Lin HK, Kan PY, Xie S, Tsai MY, Wang PH, Chen YT & Chang C 2003*b* Interleukin-6 differentially regulates androgen receptor transactivation via PI3K-Akt, STAT3, and MAPK, three distinct signal pathways in prostate cancer cells. *Biochemical and Biophysical Research Communications* **305** 462–469.

Zerbini LF, Wang Y, Cho JY & Libermann TA 2003 Constitutive activation of nuclear factor κB p50/p65 and Fra-1 and JunD is essential for deregulated interleukin 6 expression in prostate cancer. *Cancer Research* **63** 2206–2215.

Zhang L, Charron M, Wright WW, Chatterjee B, Song CS, Roy AK & Brown TR 2004 Nuclear factor-κB activates transcription of the androgen receptor gene in Sertoli cells isolated from testes of adult rats. *Endocrinology* **145** 781–789.

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