PERGAMON

Secretion of endogenous kallikreins 2 and 3 by androgen receptor-transfected PC-3 prostate cancer cells

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Abstract

Androgen independent PC-3 cells lack androgen receptor (AR) expression and do not produce kallikrein 2 (hK2) or 3 (prostate-specific antigen, PSA). In this paper, we examined the ability of androgens to stimulate PSA and hK2 production in AR transfected PC-3 cells (PC-3(AR)) and compared this to LNCaP cells. PSA and hK2 were measured in the culture medium and cell lysates using an ELISA-based immunofluorometric assay. Only androgens were able to induce PSA and hK2 secretion in PC-3(AR) cells in a dose- and time-dependent manner depending on the level of AR present. The level of androgen-induced PSA and hK2 secretion in PC-3(AR) cells was approximately 1.5 and 0.9% that induced in LNCaP cells, respectively. Insulin-like growth factor-I (IGF-I), which has been shown to activate AR in the absence of ligand, did not activate PSA secretion in the absence of androgen, but further increased the dihydrotestosterone-induced PSA secretion in PC-3(AR) cells. The lack of PSA and hK2 production in parental PC-3 cells is thus a result of their lack of AR expression. PSA and/or hK2 production in PC-3(AR) cells can thus serve as an endogenous reporter system to investigate AR action or to screen putative endocrine disrupters.
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1. Introduction

Human kallikrein 3 (hK3), more commonly known as prostate-specific antigen (PSA), and human kallikrein 2 (hK2) are androgen-regulated serine proteases secreted by prostatic glandular epithelial cells into the seminal fluid where they ultimately play a role in liquefaction of the ejaculate [1]. hK2 is present at about 1% of the concentration of PSA in seminal plasma and acts to cleave the proPSA zymogen to liberate active enzyme [2–5]. Malignant prostate epithelial cells also secrete PSA, which is detectable in serum and is widely used as a screening tool for the early detection of prostate cancer as well as to monitor for recurrence of the disease. Recent findings indicate that serum hK2 testing using highly sensitive immunofluorometric assays, could serve as a useful adjunct to improve specificity of PSA testing for distinguishing individuals with prostate cancer from those with other underlying pathologies [6,7].

Rather than acting merely as surrogate markers of prostatic disease, emerging evidence indicates that PSA and hK2 have multiple actions that may affect cancer progression. The expression of PSA as well as hK2 is not restricted to the prostate, having been detected in other cancers, including breast, lung, and uterine cancer, and in the normal breast and pituitary gland [8–12]. Wang et al. [13] have proposed that PSA acts as a mitogen in androgen responsive human prostate cancer LNCaP cells and have further postulated that the 5α-reductase inhibitor finasteride may inhibit prostate cancer growth, in part, by decreasing PSA production. Conversely, Fortier et al. [14] have demonstrated that PSA inhibits the growth and migration of endothelial cells in vitro and decreases the formation of lung metastases in mice inoculated with murine melanoma cells. They further hypothesize that the expression of PSA in prostate cancer may actually slow the progression of the disease through this antiangiogenic activity. hK2 has trypsin-like specificity and has been shown to cleave insulin-like growth factor binding

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proteins [15] and urokinase-type plasminogen activator [16], both of which could affect disease progression. Further study into the regulation of PSA and hK2 expression and function are necessary to clarify their role in prostate cancer and to better define their utility as prognostic indicators.

The regulation of endogenous PSA expression has been most widely studied in LNCaP cells; however, these cells express an androgen receptor (AR) with a threonine to alanine substitution at position 868 within the ligand-binding domain [17]. This substitution has resulted in a less discriminating receptor with regard to ligand binding and thus may not truly reflect PSA regulation. Two other human prostate cancer cell lines, DU-145 and PC-3, which are often used as models of androgen independent prostate cancer, lack AR expression and do not produce PSA or hK2 [18–20].

We have previously transfected PC-3 cells with a full-length human AR cDNA driven by a CMV promoter and have clonally isolated several stable transfectants [21]. Paradoxically, these cells undergo cell cycle arrest after treatment with androgen and ultimately undergo cell death upon prolonged or repeated androgen treatment. In this study, we present evidence that these cells produce and secrete both PSA and hK2 in an androgen-dependent manner and may serve as a useful model to study PSA and hK2 regulation.

2. Materials and methods

2.1. Cell culture

PC-3 cells stably transfected with pCEP4 alone (mock-transfected cells, PC-3(M)) or containing a full-length human AR cDNA (PC-3(AR)) have been described previously [21]. LNCaP cells were obtained from American Tissue Culture Collection (Manassas, VA). All cells were grown as monolayer cultures in RPMI 1640 medium without phenol red, supplemented with 5% heat-inactivated, charcoal-stripped, fetal bovine serum and 2 mg $NaHCO_3$, 50 U penicillin, 50 μg streptomycin, and 0.625 μg amphotericin B/ml (all from Gibco-BRL, Gaithersberg, MD) at 37 °C in a humidified CO_2 incubator. Hygromycin B (Gibco-BRL; 100 μg/ml) was added to all PC-3(AR) and PC-3(M) cultures to maintain selection. 5α-Androstan-17β-ol-3-one (dihydrotestosterone, DHT), Δ^4-androsten-17β-ol-3-one (testosterone), 1,3[10]-estratriene-3,17β-diol (estradiol), 9α-fluoro-16α-methylprednisolone (dexamethasone), 4-pregnen-3,20-dione (progesterone) and insulin-like growth factor-I (IGF-I) were obtained from Sigma Chemical Co. (St. Louis, MO). Methyltrienolone (R1881) was obtained from New England Nuclear (Boston, MA). Hydroxyflutamide and bicalutamide were gifts from Schering-Plough Research Institute (Kenilworth, NJ) and Astra-Zeneca (London, England), respectively. All compounds were dissolved in 100% ethanol and diluted with culture medium before addition to cell cultures (final ethanol content = 0.01%).

2.2. Androgen binding assays

Untreated cells were harvested using a cell scraper, homogenized in cold (4 °C) assay buffer (10 mM Tris, 1.5 mM EDTA, 10% (v/v) glycerol, 1.0 mM dithiothreitol, and 25 mM sodium molybdate, pH 7.4), and centrifuged at $102,000 \times g$ for 45 min at 4 °C. The resulting supernatant (cytosol extract) was diluted with assay buffer to 1.0 mg cytosol protein/ml. Saturation binding assays or competition binding assays were performed as described by Hoyte et al. [22] using [^{3}H]R1881 (New England Nuclear, sp. act. = 75.2 Ci/mmol) as ligand. Data were analyzed using a computer-assisted non-linear curve fitting method (LIGAND) adapted to an IBM-PC microcomputer [23].

2.3. PSA and hK2 analysis

Concentrations of PSA and hK2 were determined using ELISA-type immunofluorometric assays as previously described [24,25]. The detection limits of these assays were ≤0.001 μg/l for PSA and ≤0.006 μg/l for hK2. Secreted PSA and hK2 levels are expressed as ng/l of culture medium. PSA measured in cell lysates, prepared as described previously [26], are expressed as ng/μg or pg of total protein.

2.4. Cell proliferation assay

Cell proliferation was determined by the 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma Chemical Co.) dye-reduction method [27]. Cells were plated (5000 cells per well) into 96-well microtiter plates and treated as indicated for each experiment. On the day of the assay, 30 μl of 5 mg/ml of MTT dye were added to each well and the cells were incubated at 37 °C for 4 h. The medium was aspirated and 100 μl of dimethyl-sulfoxide (DMSO) were added to each well. Absorbance (relative optical density) was measured at 570 nM with a μQuant microplate spectrophotometer (Bio-Tek, Instruments, Winooski, VT).

2.5. Statistics

Data obtained in PSA and hK2 assays are expressed as the mean ± S.E.M, and were subjected to analysis of variance (ANOVA) followed by a Fishers LSD post-hoc test ($\alpha = 0.05$) using SSPS version 10 software for Microsoft Windows (Chicago, IL). MTT assays were analyzed using two-way ANOVA followed by Student–Newman–Keuls multiple comparison test ($P < 0.05$).

3. Results

3.1. Androgen induced PSA and hK2 secretion by PC-3 cells

Four clonally selected PC-3(AR) cell lines were chosen to determine if the lack of PSA and hK2 secretion in PC-3 cells

Table 1
Comparison of the dissociation constant (K_d) and androgen binding capacity (B_{max}) measured in cytosol extracted from clonally selected PC-3 cells stably transfected with a human AR cDNA

	K_d (nM)	B_{max} (fmol/mg protein)
PC-3(AR)$_2$	0.08	188.3
PC-3(AR)$_{13}$	0.09	235.2
PC-3(AR)$_{12}$	0.16	504.6
PC-3(AR)$_6$	0.10	604.5
LNCaP	0.18	189.3

Saturation binding assays were performed on cytosol extracts using [^{3}H]R1881 as ligand. Scatchard analysis was performed using a computer-assisted non-linear curve fitting method adapted to an IBM-PC microcomputer.

is due to the absence of endogenous AR expression. Two of the clones, PC-3(AR)$_2$ and PC-3(AR)$_{13}$, expressed moderate levels of AR, whereas the other two clones, PC-3(AR)$_6$ and PC-3(AR)$_{12}$, expressed high levels of AR as determined by saturation binding analysis (Table 1). Cells transfected with the empty pCEP4 vector (mock-transfected) were included as controls. Cells were seeded into 24-well plates and treated with 10 nM DHT or the ethanol diluent (vehicle control). Cells were harvested after 3, 7, 10, or 14 days of culture and the culture medium was assayed for PSA and hK2 content.

All four PC-3(AR) cell sublines secreted PSA and hK2 following DHT treatment with PSA levels 3–12-folds higher than hK2 (Fig. 1). PC-3(AR)$_2$ and PC-3(AR)$_{13}$ cells produced nearly equivalent levels of PSA and hK2, while PC-3(AR)$_{12}$ cells produced approximately twice as much PSA as these sublines but equivalent levels of hK2. PC-3(AR)$_6$ cells secreted more than twice the amount of PSA and hK2 as did PC-3(AR)$_{12}$ cells. Secretion of PSA or hK2 was not detected in PC-3(M) cell cultures or in PC-3(AR) cell cultures treated with vehicle (data not shown). These results demonstrate that PC-3 cells produce these kallikreins upon expression of AR and treatment with DHT. Moreover, the levels of secretions were greater in sublines that express higher levels of AR. All further studies were performed on PC-3(AR)$_2$ cells as representative of the PC-3(AR) cells.

3.2. Steroid specificity and dose relationship of PSA and hK2 secretion

The steroid specificity and dose–response relationship for PSA and hK2 secretion was studied using PC-3(AR)$_2$ cells treated with different concentrations (0.01, 0.1, 1 or 10 nM) of the natural androgens testosterone and DHT, the synthetic androgen R1881, progesterone, estradiol-17β, or dexamethasone. Control cells were treated with the ethanol vehicle. Cells were harvested 3 or 7 days after treatment and the culture media was assayed for PSA and hK2.
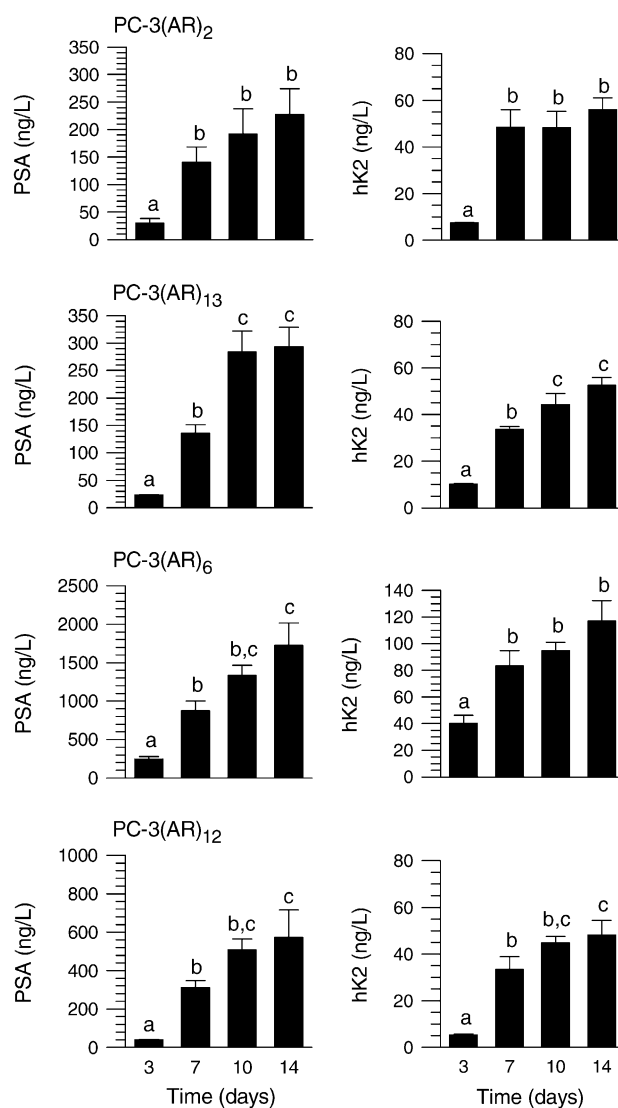

Fig. 1. PSA and hK2 levels measured in the culture medium of clonally selected PC-3 cells stably transfected with a human AR cDNA. Cell cultures were treated with 10 nM DHT or the diluent (vehicle) and culture medium was collected 3, 7, 10, or 14 days later and assayed for PSA and hK2 using an immunofluorometric assay. Data shown are for the DHT-treated cultures as PSA was not detected in medium from vehicle treated cultures. Bars represent the mean ± S.E.M. of three independent cell cultures. Within each cell line, bars with different letters are statistically different from one another as determined by a protected LSD test.

PC-3(AR)$_2$ cells treated with androgens exhibited both a time- and dose-dependent increase in PSA and hK2 secretion (Fig. 2). The highest dose (10 nM) of all the three androgens stimulated the secretion of similar levels of PSA while DHT treatment yielded slightly higher levels of hK2 than treatment with R1881 or testosterone. A dose of 1 nM R1881 resulted in a higher level of PSA than did 1 nM DHT or testosterone at both 3 and 7 days. Moreover, a higher level of PSA was measured in cells treated with 0.1 nM R1881 than DHT or testosterone at Day 7. No PSA or hK2 was detected
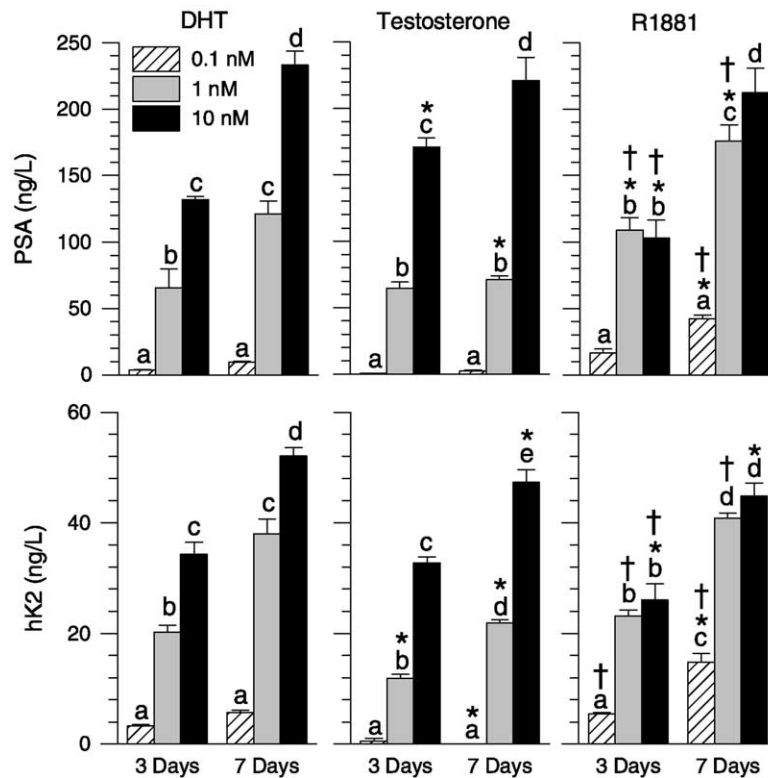
Fig. 2. Dose-dependent androgen induction of PSA and hK2 secretion in PC-3(AR)$_2$ cells. PC-3(AR)$_2$ cells were treated with 0.01–10 nM (final concentration) DHT, testosterone, R1881, or the diluent (vehicle) on Day 0. Medium was collected on Day 3 or 7 and assayed for PSA and hK2 content. Data shown are for 0.1–10 nM androgen as no PSA or hK2 was detected in medium from cultures treated with vehicle or 0.01 nM androgen. Bars represent the mean ± S.E.M. of three independent cell cultures. Within each panel, bars with different letters are statistically different from one another as determined by a protected LSD test; (*) indicates a significant difference compared to that obtained with DHT at the same dose and time; (†) indicates a significant difference between testosterone and R1881 treatment at the same dose and time.

in medium from cells treated with the lowest dose (0.01 nM) of R1881, testosterone or DHT or from cells treated with (0.01, 0.1, 1 or 10 nM) progesterone, estradiol-17β, dexamethasone, or vehicle alone; therefore, these data are not shown in the figure.

3.3. Androgen-induced PSA and hK2 secretion in LNCaP cells

The level of PSA and hK2 produced by LNCaP cells in response to different concentrations of DHT (0.01, 0.1, 1, 10 nM) was determined for comparison to PC-3(AR) cells. Cells were harvested 1, 3 or 7 days after treatment and the culture medium was assayed for PSA and hK2. DHT stimulated both a time- and dose-dependent increases in PSA and hK2 secretion with maximal hK2 levels approximately 20% that of PSA levels (Fig. 3). At the highest dose of DHT (10 nM), a 64-fold higher concentration of PSA and a 114-fold higher concentration of hK2 was measured in LNCaP cells relative to PC-3(AR)$_2$ cells (Fig. 3 versus Fig. 2). Unlike AR-transfected PC-3 cells, LNCaP cells treated with vehicle alone secreted significant levels of PSA (∼200 ng/l).

3.4. Inhibition of androgen-induced PSA and hK2 secretion in PC-3(AR)$_2$ cells by AR antagonists

The effect of AR antagonists hydroxyflutamide (HF) and bicalutamide (BiC) on PSA and hK2 secretion was examined in PC-3(AR)$_2$ cells. Cells were treated with 0.01–1 μM HF or BiC in the presence of 1 nM DHT or vehicle. PSA and hK2 levels were measured in the culture medium 3 or 7 days after treatment. HF inhibited DHT-induced PSA and hK2 secretion at the highest dose tested (Fig. 4A and C) while BiC resulted in a statistically significant reduction in PSA secretion at both 0.1 and 1 μM. Although there was a trend for reduced hK2 secretion at the highest dose of BiC, this did not attain statistical significance. Neither PSA nor hK2 secretion was detected in cells treated with vehicle, HF, or BiC alone (data not shown).

As DHT inhibits the proliferation of PC-3(AR) cells, the effect of HF and BiC on growth of PC-3(AR)$_2$ cells was determined in the presence or absence of 1 nM DHT. As expected, DHT inhibited the growth of these cells (Fig. 5A and B). While HF or BiC treatment alone had no effect (Fig. 5A and B), the highest doses of these antagonists (1.0 μM) significantly reversed the growth-inhibitory actions of DHT
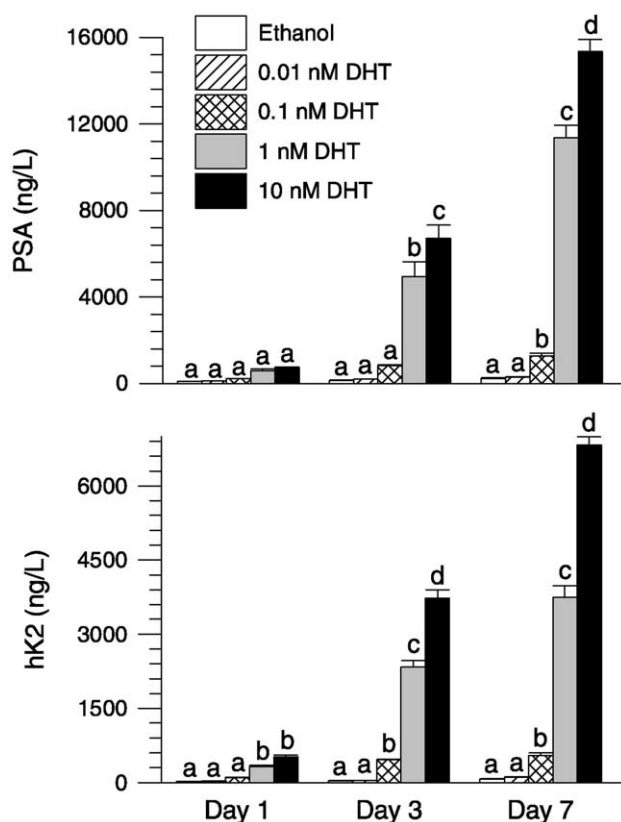
Fig. 3. Dose-dependent androgen induction of PSA and hK2 secretion in LNCaP cells. LNCaP cells were treated with 0.01–10 nM (final concentration) DHT or the diluent (vehicle) on Day 0. Medium was collected on Day 1, 3 or 7 and assayed for PSA and hK2 content. PSA protein was also detected in medium from LNCaP cells treated with vehicle. Bars represent the mean ± S.E.M. of three independent cell cultures. Within each time point, bars with different letters are statistically different from one another as determined by a protected LSD test.

(Fig. 5A and B). These data confirm that these ligands act as AR antagonists in these cells and that the reduction in DHT-induced PSA secretion in PC-3(AR)$_2$ cells by HF or BiC treatment is not due to a reduction in cell number.

3.5. Reduced PSA production and secretion following DHT removal

Our data demonstrate that androgens increase PSA and hK2 secretion in androgen sensitive PC-3 cells, albeit at much lower levels than LNCaP cells. The accumulation of these kallikreins in the culture medium increased steadily during the first 7 days following DHT exposure, perhaps reflecting continued androgen action. To determine the effect of restricting DHT exposure on PSA production and secretion, the culture medium of PC-3(AR)$_2$ cells treated with 10 nM DHT was replaced with DHT-free medium after 24 h. Cells in which the medium was not replaced were included as controls, as were vehicle-treated cells. In addition, the effect of changing the culture medium again on

Day 3 was assessed to determine if nutrient replenishment increased PSA production by these cells. Cell lysates and culture medium were collected at 1, 3 or 7 days after DHT treatment and assayed for PSA content. Similarly, treated LNCaP cells were included for comparison.

PSA accumulated in the culture medium increased steadily from Day 1 to 7 in both PC-3(AR)$_2$ and LNCaP cells in the absence of medium changes (Fig. 6A and C). While an increase in PSA content in cell lysates was observed from Day 1 to 3, no further increase was noted from Day 3 to 7 in both the cell lines (Fig. 6B and D). Removal of DHT from LNCaP and PC-3(AR)$_2$ cell cultures after 24 h (medium change at Day 1) resulted in reduced PSA secretion measured at Day 3 and 7 compared to PSA accumulated in the culture medium in the absence of medium changes (Fig. 6A and C). While a significant decrease in PSA content in LNCaP cell lysates was observed from Day 3 to 7 in cultures with DHT removed after 24 h, little or no decrease was noted in similarly treated PC-3(AR)$_2$ cells (Fig. 6B and D). Changing the culture medium again at Day 3 decreased further the secreted PSA levels in both cell lines at Day 7 (Fig. 6A and C). This decrease in PSA production and secretion was more pronounced in LNCaP compared to PC-3(AR)$_2$ cells (Fig. 6).

3.6. Stimulation of androgen-induced PSA secretion in PC-3(AR)$_2$ cells by insulin-like growth factor-I (IGF-I)

Ligand independent activation of the AR by IGF-I has been reported in transfection studies using androgen responsive reporter genes [28,29]. The ability of IGF-1 treatment to stimulate PSA secretion in PC-3(AR)$_2$ cells was thus examined. Cells were treated with 25 or 50 ng/ml of IGF-I in the presence or absence of 1 or 10 nM DHT, and PSA levels were measured in the culture medium 1, 3 or 7 days after treatment. PSA secretion was not detected in cells treated with vehicle or IGF-I alone (data not shown); however, IGF-I enhanced DHT-induced PSA secretion in a dose- and time-dependent manner (Fig. 7A and B). Cell proliferation assays indicated that IGF-I had no effect on PC-3(AR)$_2$ cell growth and did not alter DHT-induced growth inhibition in these cells (data not shown).

4. Discussion

The results presented in this study demonstrate that PC-3 cells have the ability to produce and secrete endogenous PSA and hK2 when they are transfected with a full-length human AR cDNA. The production and secretion of these kallikreins was induced by androgens in a dose- and time-dependent manner but not by progestins, estrogens or glucocorticoids. The inhibition of the androgen-induced production and secretion of both kallikreins by AR antagonists further verified the specificity of this androgen response. Previous studies have shown LNCaP cells secrete
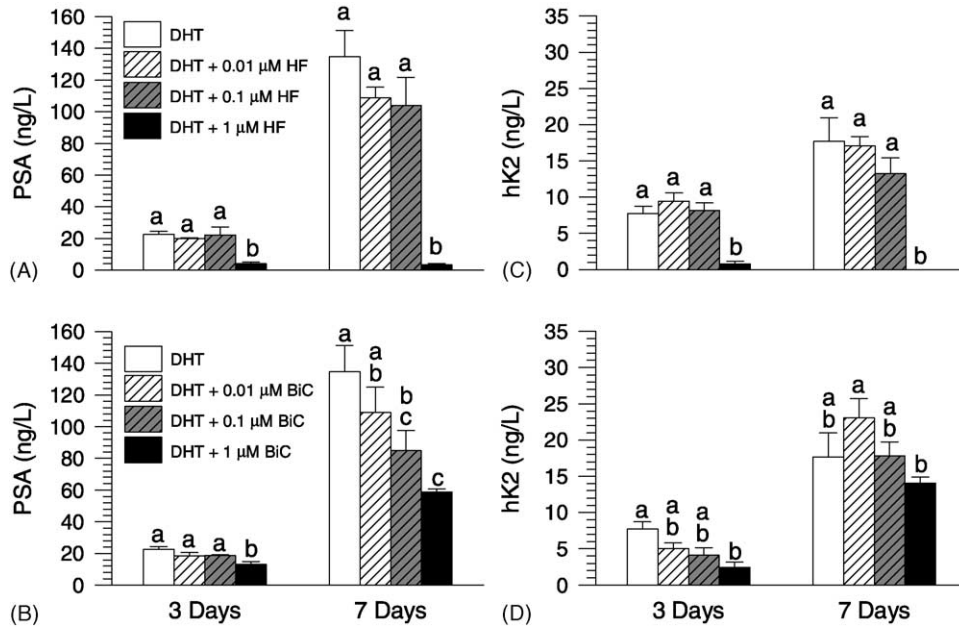
Fig. 4. Inhibition of DHT-induced PSA and hK2 secretion in PC-3(AR)$_2$ cells by hydroxyflutamide (HF) and bicalutamide (BiC). PC-3(AR)$_2$ cells were treated with: (A and C) 0.01–1.0 μM HF; or (B and D) BiC in the presence or absence of 1.0 nM DHT. Vehicle-treated cells were included as a control. Culture medium was collected 3 or 7 days after treatment and assayed for PSA and hK2 content. PSA or hK2 was not detected in medium from cultures treated with vehicle, HF, or BiC only; therefore, only data from cultures treated with DHT are shown. Bars represent the mean ± S.E.M. of three independent cell cultures. Within each panel and time point, bars with different letters are statistically different from one another as determined by a protected LSD test.
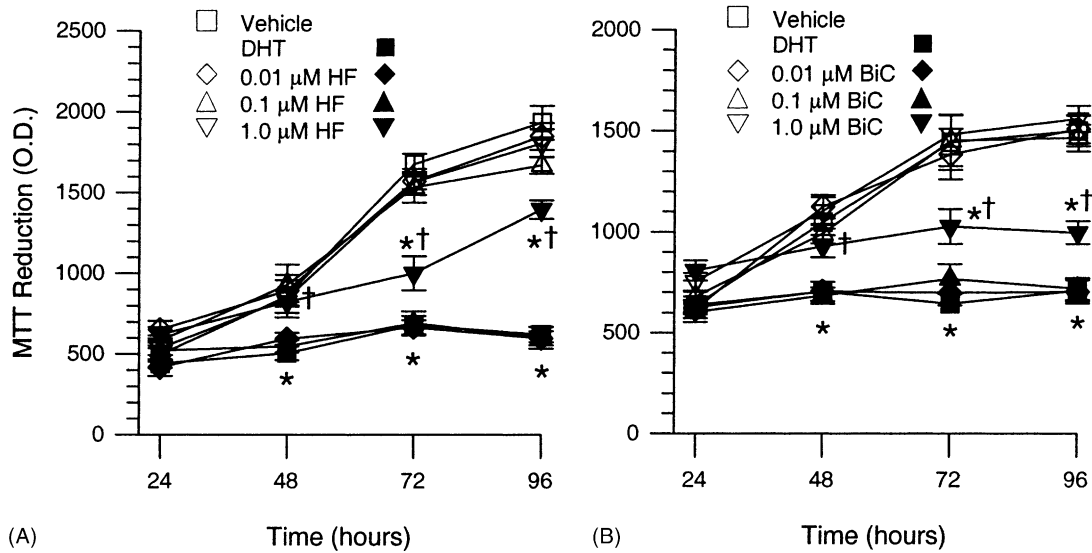

Fig. 5. Effect of HF and BiC on the growth of PC-3(AR)$_2$ cells. PC-3(AR)$_2$ cells were treated with: (A) 0.01–1 μM HF; or (B) BiC in the presence or absence of 1.0 nM DHT. Cell proliferation was measured by the MTT dye reduction assay every 24 h. Data are expressed as mean ± S.E.M. of eight replicates per point and were analyzed by two-way ANOVA followed by Student–Newman–Keuls post-hoc multiple comparison test; (*) indicate a significant difference relative to vehicle (ethanol)-treated cells; (†) indicate a significant difference relative to DHT treated cells; open symbols represent vehicle-treated cells, whereas filled symbols represent DHT-treated cultures.

PSA after stimulation with androgens, progestins and estradiol [30]. While this may reflect the ability of these steroids to activate the mutated AR expressed by LNCaP cells, several breast cancer cell lines have also been shown to secrete PSA and hK2 following treatment with androgen, progestin,

glucocorticoid and estrogen [31–33]. The lack of progesterone or estradiol effects in PC-3(AR) cells may be due to lack of estrogen receptor and progestin receptor expressions [34,35]; however, Lau et al. [36] have detected estrogen receptor α and β mRNA expression in PC-3 cells by RT-PCR.
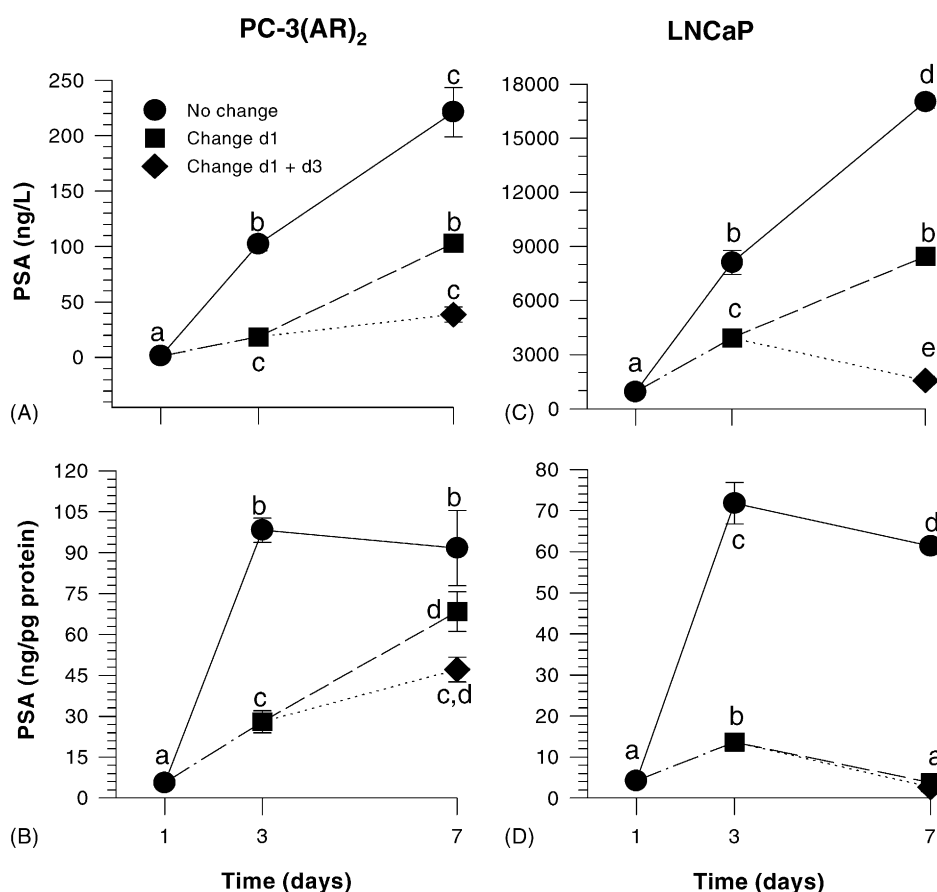
Fig. 6. Effect of cell culture medium changes on PSA production and secretion by PC-3(AR)$_2$ and LNCaP cells. PC-3(AR)$_2$ and LNCaP cells were treated with 10 nM DHT (final concentration) or diluent (vehicle) on Day 0. Culture medium and cells were harvested on Day 1, 3, or 7 and PSA levels were determined for both: (A and C) the medium; and (B and D) cell lysates. In some cultures, the medium was changed: (d1) at 24 h; or (d1 and d3) at 24 and 72 h, to determine the effect upon PSA production and secretion. Only data from DHT-treated cultures is shown as PSA was not detected in the absence of DHT treatment in PC-3(AR)$_2$ cells. Bars represent the mean ± S.E.M. of 3–9 independent cell cultures. Within each panel, points with different letters are statistically different from one another as determined by a protected LSD test.

PC-3 cells express glucocorticoid receptor and respond to dexamethasone with decreased growth [37]; thus the lack of dexamethasone-induced PSA secretion suggests that the glucocorticoid receptor, at least in these cells, does not regulate expression of either *hKLK2* or *hKLK3*, which encode hK2 and PSA, respectively. Our results also indicate that production of PSA and hK2 in PC-3(AR) cells requires treatment with androgen, whereas LNCaP cells secrete significant levels in the absence of exogenous steroid hormone treatment.

Two previous studies [38,39] have suggested that AR-transfected PC-3 cells express low levels of PSA mRNA detectable by RT-PCR; however, no indication of protein production or direct comparison with LNCaP cells was provided. The levels of PSA and hK2 secretion in PC-3(AR) cells in the present study were proportional to the levels of AR expression measured in the cells. However, the secretion of PSA and hK2 was substantially lower than that measured in LNCaP cells. As PC-3(AR)$_6$ and PC-3(AR)$_{12}$ cells express higher levels of AR than do LNCaP cells [21], this difference can not be attributed to lower levels of AR

expression. Recent studies indicate that PC-3 and LNCaP cells possess different expression profiles of nuclear receptor co-activators [40,41]. In addition, Gross et al. [42] have recently reported that protein inhibitor of activated STAT (PIAS) family members, which interact with the ligand binding domain of the AR, modulate the effects of androgen on PSA production in LNCaP cells, with PIASy acting as a repressor and PIAS1 acting as an activator. It remains to be determined if differential expression of co-activators and -repressors in PC-3 and LNCaP cells contribute to the differences in *hKLK2* and *hKLK3* expression.

The differences in *hKLK2* and *hKLK3* expression between LNCaP and PC-3(AR) cells could also be due to unknown modifications within the promoter and enhancing regions of these genes. The gene *hKLK2* is located 12 kb downstream of *hKLK3* on chromosome 19q [43,44]. Two androgen response elements (AREs) have been identified in the 5 kb 5′ flanking region of *hKLK2* [45,46] and at least three AREs have been identified in the 5 kb 5′ flanking region of *hKLK3*—two (AREs I and II) in the proximal promoter and a third (ARE
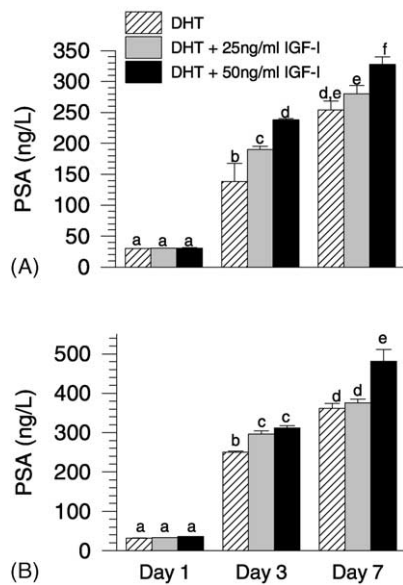
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Fig. 7. Stimulation of DHT-induced PSA secretion in PC-3(AR)$_2$ cells by insulin-like growth factor I (IGF-I). PC-3(AR)$_2$ cells were treated with 25 or 50 ng/ml IGF-I in the presence or absence of: (A) 1.0 nM DHT; or (B) 10 nM DHT. Vehicle-treated cells were included as a control. Culture medium was collected 3 or 7 days after treatment and assayed for PSA content. PSA was not detected in medium from cultures treated with vehicle or IGF-1 only and are, therefore, not shown. Bars represent the mean ± S.E.M. of three independent cell cultures. Within each panel and time point, bars with different letters are statistically different from one another as determined by a protected LSD test.

III) in the enhancer region [47]. The presence of ARE III in the PSA enhancer region is sufficient and necessary for PSA production while the presence of this response element together with ARE I resulted in maximal PSA stimulation in LNCaP cells [47]. Huang et al. [48] more recently reported the presence of at least four non-consensus AREs surrounding ARE III that act synergistically to augment androgen activation of PSA expression. In addition, this region binds as yet unidentified transcription factors or proteins that play an important role in regulating PSA production [49]. Interestingly, footprinting studies indicate protein binding to a region of 15 bp 5′ to ARE III in LNCaP cells but not in PC-3 cells [49]. This difference might, therefore, contribute to the lower PSA production in PC-3(AR) cells as compared to LNCaP cells.

Activation of AR in a ligand independent manner has been reported for several growth factors, including IGF-I and -II, epidermal growth factor, and keratinocyte growth factor [28,29], interleukin-6 [50,51] in LNCaP or AR-transfected DU145 prostate cancer cells, using reporter gene constructs. Nazareth and Weigel [52] have also shown that forskolin, an activator of the protein kinase A signaling pathway, is also able to activate AR by a ligand independent mechanism in transfected PC-3 cells. PC-3 cells express IGF-I receptors [53] and secrete IGF-binding proteins [54,55]. Our data indicate that IGF-I treatment does not induce PSA secretion in

PC-3(AR)$_2$ cells; however, it had a synergistic effect when combined with DHT treatment. This suggests that ligand independent activation of the AR by IGF-I is insufficient to induce PSA expression in PC-3(AR) cells. It is presently not clear how IGF-I may act to enhance androgen-induced PSA secretion in these cells.

These findings demonstrate and characterize endogenous PSA and hK2 protein production and secretion in PC-3 cells stably transfected with a full length AR cDNA. The loss of expression of both these kallikreins in the parental cell line is, therefore, due to the lack of AR expression. However, other factors may also be involved as the levels of hK2 and PSA induced by DHT were a fraction of that measured in LNCaP cells. PSA and/or hK2 production can thus serve as an endogenous reporter system in PC-3(AR) cells to examine AR action.

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