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Prostate. Author manuscript; available in PMC 2014 April 21.

Published in final edited form as:
Prostate. 2014 February ; 74(3): 235–249. doi:10.1002/pros.22745.

5α-ReductaseType 3 Enzyme in Benign and Malignant Prostate

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Abstract

BACKGROUND—Currently available 5α-reductase inhibitors are not completely effective for treatment of benign prostate enlargement, prevention of prostate cancer (CaP), or treatment of advanced castration-recurrent (CR) CaP. We tested the hypothesis that a novel 5α-reductase, 5α-reductase-3, contributes to residual androgen metabolism, especially in CR-CaP.

METHODS—A new protein with potential 5α-reducing activity was expressed in CHO-K1 cellsandTOP10 *E. coli* for characterization. Protein lysates and total mRNA were isolated from preclinical and clinical tissues. Androgen metabolism was assessed using androgen precursors and thin layer chromatography or liquid chromatography tandem mass spectrometry.

RESULTS—The relative mRNA expression for the three 5α-reductase enzymes in clinical samples of CR-CaP was 5α-reductase-3 ≫ 5α-reductase-1 >5α-reductase-2. Recombinant 5α-reductase-3 protein incubations converted testosterone, 4-androstene-3,17-dione (androstenedione) and 4-pregnene-3,20-dione (progesterone) to dihydrotestosterone, 5α-androstan-3,17-dione, and 5α-pregnan-3,20-dione, respectively. 5α-Reduced androgen metabolites were measurable in lysates from androgen-stimulated (AS) CWR22 and CR-CWR22 tumors and clinical specimens of AS-CaP and CR-CaP pre-incubated with dutasteride (a bi-specific inhibitor of 5α-reductase-1 and 2).

CONCLUSION—Human prostate tissues contain a third 5α-reductase that was inhibited poorly by dutasteride at high androgen substrate concentration in vitro, and it may promote DHT formation in vivo, through alternative androgen metabolism pathways when testosterone levels are low.

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Keywords

5α-reductase; androgen metabolism; dihydrotestosterone; benign prostatic hyperplasia; castration-recurrent prostate cancer

INTRODUCTION

The 5α-reductase family of enzymes play a critical role in the biosynthesis of androgens by catalyzing the irreversible conversion of testicular androgen, testosterone, to dihydrotestosterone (DHT), or the adrenal androgen, androstenedione, to 5α-androstan-3,17-dione (5α-ASD). In the prostate, 5α-reductase-2 catalyzes the intracrine target tissue based metabolism of testosterone to DHT, the most potent ligand for androgen receptor (AR). AR occupied by DHT transduces the androgenic signal into a gene expression pattern essential for normal prostate development and function of mature prostate epithelium. The finding that DHT, not testosterone, is the preferred ligand for driving AR-mediated transactivation in the prostate led to multiple therapeutics that seek to manipulate the androgen-AR signaling pathway [1].

Two structurally similar azasteroid molecules based on the pregnane C_{21} backbone, finasteride, and dutasteride, were designed as mechanism-based inhibitors of 5α-reductase [2] and are major pharmacological tools for clinical management of prostate disease. Finasteride is a specific 5α-reductase-2 inhibitor that decreased serum DHT levels by ~70% [3] and intra-prostatic DHT levels by 85% [4] in men with benign prostate (BP) enlargement. Dutasteride inhibited both 5α-reductase-1 and -2 at physiologically attainable concentrations and suppressed DHT production to a greater extent than finasteride. Dutasteride decreased serum levels of DHT by ~95% [5] and suppressed intraprostatic DHT levels by >95% [6] in men with BP enlargement.

Two large clinical studies evaluated finasteride or dutasteride for prostate cancer (CaP) chemoprevention. The Prostate Cancer Prevention Trial demonstrated a 25% reduction in risk of developing CaP in men who received finasteride [7]. Similarly, men treated with dutasteride in the Reduction by Dutasteride of CaP Events study exhibited a 23% risk reduction of CaP [8]. Furthermore, in a pilot study, dutasteride treatment of men with early-stage CaP resulted in a measureable decrease in prostate volume due to prostate epithelial apoptosis [9], suggesting that 5α-reductase-1 activity is crucial in androgen-stimulated (AS) CaP. However, interpretation of the results from those two clinical studies remains controversial because diagnosis of CaP is delayed until high-grade aggressive CaP disease occurs [10].

Evaluation of the therapeutic effects of finasteride or dutasteride in treatment of advanced CaP during androgen deprivation therapy (ADT) demonstrated that men derived limited or no benefit from the inhibition of 5α-reductase-1 and -2 activity. Men given finasteride (10 mg daily) were found to have a delayed increase in serum PSA, but no difference in local recurrence or distant metastasis [11]. Similarly, dutasteride therapy produced a biochemical response in a limited number of men, although their disease, progressed during ADT to castration-recurrent (CR) CaP [12]. These clinical studies and laboratory research using dual

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5α-reductase-1 and -2 inhibitors or double knockout (both 5α-reductase-1 and -2)mice [13–16] suggested the potential presence of additional 5α-reductase activity in AS-CaP, and CR-CaP. Herein, we demonstrate that both recombinant 5α-reductase-3 expressed in mammalian or bacterial systems and endogenous 5α-reductase-3 in homogenates of preclinical and clinical prostate specimens are capable of 5α-reduction of 3-keto-Δ4 precursor androgens and that this activity is not inhibited by dutasteride or abiraterone (a cytochrome P450 17α-hydroxylase 17/20 lyase [CYP17A1] inhibitor) at high substrate concentrations.

MATERIALS ANDMETHODS

Cell Culture Reagents

All cell lines were purchased from ATCC (www.atcc.org). The cell lines CV-1 (ATCC-CCL-70), COS, and HEK 293 (ATCC-CRL-1573) cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Mediatech Cellgro, Herndon, CA) and CHO-K1 cells (ATCC-CCL-61) were cultured in Ham's F-12K medium (Cellgro) supplemented with 10% FBS at 37°C. Anti-6X-His and anti-Thio antibodies, and pBAD/Thio-TOPO vector and Positope molecular weight markers were purchased from Life Technologies Corp./Invitrogen (Grand Island, NY). Peroxidase-conjugated, affinity purified goat anti-rabbit, or goat anti-mouse IgG were purchased from Jackson Immuno Research Laboratories, Inc. (West Grove, PA). All unlabeled steroid substrates and steroid standards were purchased from Steraloids, Inc. (Newport, RI). Radiolabeled 4-androsten-17β-ol-3-one (testosterone), 4-androstene-3,17-dione (androstenedione), and 4-pregnene-3,20-dione (progesterone)were purchased from PerkinElmer, Inc. (Waltham, MA). Abiraterone was purchased from Selleck Chemicals (Houston, TX). Dutasteride was a gift from Roger Rittmaster, M.D. (GlaxoSmithKline, Research Triangle Park, NC).

Plasmid Construction

A 5α-reductase-3 cDNA (gift of Juergen Reichardt, Ph.D., University of Sydney, Camperdown, New South Wales, Australia) was cloned into the PET100/D expression vector using BglII and SacI endonucleases (New England Biolabs, Inc., Ipswich, MA). An 1,183-bp fragment that encoded 5α-reductase-3 with an N-terminal polyhistidine (6X-His)/X-press epitope tag was subcloned into pDC316 (Microbix Biosystems Inc., Mississauga, Ontario, Canada) predigested with BglII and SacI to generate an adenovirus shuttle plasmid. Subsequently, the 5α-reductase-3 sequence from the PET100/D-5α-reductase-3 vector was PCR amplified and cloned into the pBAD/Thio-TOPO vector using the TOPO cloning reaction (pBAD/TOPO ThiofusionExpression Kit; Life Technologies Corp./Invitrogen).

Adenovirus Generation and Amplification

Adenovirus was generated by co-transfection of the pDC316 5α-reductase-3 shuttle plasmid (murine CMV promoter) and the pBH Gloxp (Microbix) vector that contained the adenovirus type-5 genome without E1 and E3 regions into HEK 293 cells using the SuperFect transfection system (Qiagen, Valencia, CA). HEK 293 cells were harvested when

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cytopathic effects appeared. Cells were lysed in three freeze-thaw cycles using a dry ice-ethanol bath and the lysate was centrifuged at 5,000 rpm for 5 min at 4°C to remove cellular debris; the supernatant was saved as a virus stock. Adenovirus (Ad-5α-reductase-3) was amplified by infecting HEK 293 cells with virus stock in tissue culture medium without serum for 3 hr and the cultures overlaid with DMEM medium containing 10% FBS. The infected cells were collected and processed when viral cytopathic effects were identified. The Ad-5α-reductase-3 titer was measured using an Adeno-X rapid titer kit (Clontech Laboratories, Inc., Mountain View, CA).

Expression of Recombinant 5α-Reductase-3 Protein

Mammalian—CHO-K1 cells (70–80% confluent in six-well plates) were infected with Ad-5α-reductase-3 in serum-free Ham's F-12K medium for 3 hr, grown in Ham's F-12K supplemented with 10% FBS for 21 hr at 37°C in 5% CO_2, and cells were harvested for immunoblot analysis and RNA extraction. Protein components from the cell lysates were resolved on 10% SDS-polyacrylamide gels and electroblotted to Immobilon-P membranes (Millipore Corp., Billerica, MA). Immunoblotting was performed using anti-6X-His antibody (Life Technologies Corp./Invitrogen), and 5α-reductase-3 protein was detected on luminescence readout (Pierce Biotechnology, Inc., Rockford, IL).

Bacterial—5α-Reductase-3 protein was expressed using the pBAD/Thio-TOPO vector in TOP10 *E. coli* cells to increase the solubility of expressed 5α-reductase-3 protein. 5α-reductase-3 protein was semipurified using a ProBond purification system (Life Technologies Corp./Invitrogen). Expressed 5α-reductase-3 protein was detected using anti-6X-His, or anti-Thio antibodies (Life Technologies Corp./Invitrogen), and confirmed identity using a specific rabbit polyclonal anti-5α-reductase-3 antibody [17].

Recombinant 5α-Reductase-3 Protein Activity

Recombinant 5α-reductase-3 enzyme activity was tested using 3-keto-Δ4 substrates: testosterone (4-androsten-17β-ol-3-one), 4-androstene-3,17-dione (androstenedione) or 4-pregnene-3,20-dione (progesterone). A 1.0 μM concentration of steroid substrate was used in all incubations to assess in vitro 5α-reductase-1, -2, and -3 activity [18]. CHO-K1 cell lysates were prepared, incubated at 37°C and 5α-reductase-3 activity was assayed using methods described previously [19]. Ad-5α-reductase-3-infected, or -uninfected control CHO-K1 cell lysates were mixed with 1.0 ml ice-cold buffer of 10 mM Tris, pH 7.8, 1.0 mM dithiothreitol, 1.0 mM fresh phenylmethylsulfonyl chloride, and 1X Complete protease inhibitor (Roche, Indianapolis, IN) and sonicated at 20% power for 10 bursts (1 sec each). Cell lysates were placed on ice, protein concentrations measured using the Bio-Rad protein assay, and enzymatic activity assayed immediately. Enzyme assay buffer for all incubations contained 100 mM Tris-citrate, 0.5 mM dithiothreitol and 1.0 mM NADPH. CHO-K1 protein lysates (250 μg) were incubated with 1.0 μCi of [^{3}H]-testosterone containing 1.0 μM testosterone at pH 4.5, 5.5, 6.5, 7.5, and 8.5 for 1 hr in a 37°C water bath to determine the optimum pH.

Analysis of inhibition of 5α-reductase-3 activity was performed using lysates in enzyme assay buffer (100 mM Tris-citrate, 0.5 mM dithiothreitol, and 1.0 mM NADPH) pre-

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incubated with dutasteride at three concentrations (7.0 nM, the IC_{50} for inhibition of 5α-reductase-1 and -2 [2]; 100 nM, the measured concentration of dutasteride in BP specimens [20]; and 150 nM), or abiraterone at two concentrations (100 and 150 nM) for 5 min at 27°C, after which substrate was added and the reaction mixture incubated as above. All incubations were quenched with 1.0 ml ice-cold chloroform/acetone (9:1, v/v), vortexed, centrifuged at 5,000 rpm for 5 min and placed on ice. The organic phase was separated from the aqueous phase, evaporated, and reconstituted in 40 μl chloroform/acetone (9:1) containing internal standards (testosterone, DHT, androstenedione and 5α-ASD; 0.2 mg/ml). Androgen samples were separated on silica-coated plates using a chloroform:acetone (9:1) mobile phase [21]. The positions of authentic standards on the developed plates were visualized using iodine vapor, and chromatographic zones corresponding to the reference standards were scraped into vials containing 500 μl ethanol. Liquid scintillation cocktail (8.0 ml Ecoscint; National Diagnostics, Atlanta, GA) was added and the levels of tritiated metabolites co-isolated with areas of testosterone, DHT, androstenedione, and 5α-ASD were quantitated using a liquid scintillation counter (Packard TC 2100TR). The amount of individual radioactive 5α-reduced products was calculated as the percentage of total [^{3}H]-radioactivity recovered in each sample lane on the thin layer chromatography plate. Uninfected control CHO-K1 cell lysates incubated with radiolabeled androgen substrate were subtracted to calculate Ad-5α-reductase-3-velocity data.

The 5α-reductase-3 enzyme activity was corroborated using semi-purified thioredoxin-5α-reductase-3 fusion protein (50 μg) incubated at 37°C with 0.5 μM unlabeled testosterone, androstenedione, or progesterone at pH 7.4. After 15 min, incubations (50 μl) were quenched, steroids extracted and each 5α-reduced metabolite, DHT, 5α-ASDor 5α-pregnan-3,20-dione (DHP), was characterized using liquid chromatography tandem mass spectrometry (LC/MSMS) [22].

Endogenous 5α-Reductase-3 Protein Activity

Preclinical human CWR22 prostate xenografts were harvested from intact mouse hosts; mouse hosts on days 40, 80, and 120 after castration; and mouse hosts with apparent CR-CWR22 xenografts at least 150 days after castration. Incubation of xenograft lysates, reaction quenching, recovery of 5α-reduced products, and thin layer chromatography separations were performed as described above. Frozen xenografts were pulverized and sonicatedon ice to prepare lysates. Analyses of 5α-reductase-3 activity were performed using 1.0 μCi [^{3}H]-androstenedione or 1.0 μCi [^{3}H]-progesterone containing 1.0 μM androstenedione or 1.0 μM progesterone, respectively.

Research samples of human AS-BP and AS-CaP tissue were procured from radical prostatectomy specimens. CR-CaP specimens were procured during trans-urethral resection of the prostate in men who experienced urinary retention from CaP recurrence during ADT. Three specimens of AS-BP, three specimens of AS-CaP as well as three CR-CaP specimens, each harvested from different patients, were combined, homogenized, and the protein concentration determined. 5α-reductase-3 activity was analyzed from AS-BP, AS-CaP, or CR-CaP lysates as described above. Procurement [23] and the experiments using human

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prostate specimens were approved by the Roswell Park Cancer Institute's Institutional Review Board.

The total endogenous 5α-reductase enzyme capability was evaluated using lysates of clinical specimens incubated with a range of dutasteride concentrations (1.0 nM to 100 μM) that included the clinically measured intraprostatic tissue levels of dutasteride (100 nM) in AS-BP [20]. The altered androgenic metabolite profiles between the AS-BP, AS-CaP and CR-CaP tissues were determined by LC/MSMS. Tissue lysates (270 μg) were mixed with 240 μl reaction buffer (pH 7.4) containing 1.0 mM NADPH and dutasteride and preincubated for 5 min at 27°C, followed by addition of unlabeled testosterone or androstenedione(1.0 μM) and incubated for 45 min at 37°C. Control incubations were performed without dutasteride. Incubations were quenched with chloroform/acetone (9:1). The organic extracts from incubations were evaporated, reconstituted in methanol/water, and levels of DHT, 5α-ASD, and 5α-androstan-3α-ol-17-one (androsterone) were quantified by LC/MSMS [22].

5α-Reductase-3 mRNA in Clinical Specimens and CWR22 Xenografts

Total RNA was isolated from tissue specimens of CR-CaP (n = 3), microdissected AS-BP (n = 3), and microdissected AS-CaP (n = 3) [24] using RNeasy Mini kits (Qiagen). In addition, RNA was isolated from AS-CWR22 xenografts from intact mouse hosts (n = 3), xenografts harvested after castration at 20 days (n = 3) and CR-CWR22 xenografts (n = 3). Total RNA (400 ng) was reverse-transcribed into cDNA using random primers and reverse transcriptase (Life Technologies Corp./Invitrogen) and the cDNA was used as template for analysis of expression of selected genes. Primers and probes for 5α-reductase-1, 5α-reductase-3, hydroxysteroid dehydrogenase-17β3 (HSD17β3) and aldo-keto reductase 1C3 (AKR1C3) target genes were purchased from Applied Biosystems (Carlsbad, CA). A primer pair and probe were synthesized for 5α-reductase-2: forward, 5′-ATTTGTGTACTCACTGCTCAATCGA-3′; reverse, 5′ AGGCAGTGCCTCTGAGAATGA-3′; and probe, 5′ FAM-AGGCCTTATCCAGCTAT-3′ MGB. PCR reactions were performed using a 7300 Real-Time PCR system (Applied Biosystems) with a total reaction mixture volume of 25 μl containing 8.0 ng cDNA, 1X TaqMan Universal PCR Master Mix (Applied Biosystems) and 1X primers and probe mix. The PCR conditions for all reactions were 95°C for 10 min, followed by 40 amplification cycles at 95°C for 15 sec and 60°C for 1 min. 5α-Reductase-1, 5α-reductase-2, and 5α-reductase-3 mRNA from clinical specimens and CWR22 xenografts was normalized to the endogenous housekeeping gene, GAPDH, and comparative RT-PCR ($2^{-\Delta\Delta C_T}$) was used to quantify mRNA levels. The relative mRNA levels in CaP specimens or CWR22 xenografts were normalized to AS-BP tissue specimens or AS-CWR22 xenograft, respectively.

RESULTS

Expression and Enzyme Activity of Recombinant 5α-Reductase-3 Protein

CHO-K1 cells were chosen for expression of recombinant 5α-reductase-3 protein because steroid 5α-reductase-1 or 5α-reductase-2 enzymatic activity is negligible [18]. Furthermore,

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5α-reductase-3 mRNA detected in untransfected CHO-K1 cells required multiple amplification cycles that resulted in primer specificity deterioration (Fig. 1A). In COS and CV1 cells, endogenous expression of both 5α-reductase-1 and -3 genes was observed, suggesting that both contributed to background 5α-reducing activity. Transfection of CHO-K1 cells with increasing quantities of adenovirus carrying an 1,183-bp fragment that encoded 5α-reductase-3 produced a dose-dependent increase in gene expression (Fig. 1B). Immunoblot analysis of cell lysates demonstrated increased levels of 5α-reductase-3 protein at the predicted molecular weight over an increasing range of adenovirus infection units (multiplicity of infection). Most of the immunoreactive 5α-reductase-3 protein synthesized by the transiently transfected CHO-K1 cells remained at the top of the SDS-PAGE gel. This 5α-reductase-3 protein aggregate was not prevented by treatment of cell lysates with inclusion body solubilization reagent (Pierce), de-glycosylating enzymes (Sigma, St. Louis, MO), trypsin or other proteases, or by decreasing the incubation time for expression (Fig. 1C). The enzymatic activity of recombinant 5α-reductase-3 present in CHO-K1 cell lysates was characterized across the pH range of 4.5 to 8.5 and the rate of enzymatic conversion of testosterone (1.0 μM) to DHT ranged from 33.1 to 59.3 pmol/mg protein/hr (Fig. 1D). The maximum conversion rate was observed at pH 6.5, but 5α-reductase-3 enzyme activity was present across the range from acidic to basic pH.

The enzymatic activity of expressed 5α-reductase-3 was examined using steroid inhibitors, dutasteride or abiraterone, and 1.0 μM testosterone as substrate at pH 7.4. CHO-K1 cell lysate preincubated with physiologic concentrations dutasteride or abiraterone partially inhibited DHT biosynthesis. In CHO-K1 cell lysate at high adenovirus infection units the rate of DHT biosynthesis was approximately 37 pmol/mg protein/hr both with and without 7.0 nM dutasteride (Fig. 2A). Uninfected CHO-K1 cell lysate in the presence of100 nM and 150 dutasteride resulted in ~36% and ~43% decrease in DHT biosynthesis, respectively (Fig. 2B). At low adenovirus infection units, the conversion of testosterone to DHT decreased to 14.5 pmol/mg protein/hr and the addition of 100 and 150 nM dutasteride reduced DHT biosynthesis ~38% and ~46%, respectively (Fig. 2C). Furthermore, abiraterone at 100 or 150 nM decreased 5α-reductase-3 activity only 20% (Fig. 2D). The levels of 5α-ASD were measured in all lysate incubations to identify possible enzyme oxidation of DHT.

A bacterial pBAD/TOPO vector expression system was used to overexpress a thioredoxin/5α-reductase-3 fusion protein, thus decreasing aggregate formation and increasing the yield of active 5α-reductase-3. The expressed chimeric 5α-reductase-3 protein was semi-purified using 6X-His-tagged beads (Fig. 3A), and the presence of 5α-reductase-3 detected with anti-6X-His (lanes 1–2, 4–5, 7–8) or anti-thioredoxin antibodies (lane 9). 5α-Reductase-3 protein inclusion bodies were detected in the bacterial pellet using a polyclonal anti-5α-reductase-3 antibody (lane 10). Semipurified 5α-reductase-3 protein (50 μg) was incubated with 3-keto-Δ4 steroid substrates(0.5 μM testosterone, androstenedione, or progesterone) to demonstrate the enzyme's activity. The 5α-reduced products, DHT (Fig. 3B), 5α-ASD (Fig. 3C), and DHP (Fig. 3D), were identified using LC/MSMS. These analyses confirmed that concentrated recombinant 5α-reductase-3 expressed in bacteria converted testosterone to DHT, adrenal androgen, androstenedione to 5α-ASD, and the cholesterol metabolite progesterone to DHP. In the absence of 5α-reductase-3

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enzyme or NADPH conversion of the 3-keto-Δ4 substrates to DHT, 5α-ASD, or DHP did not occur (data not shown).

Endogenous Expression and Enzyme Activity of 5α-Reductase-3 in CWR22 Xenografts

The mRNA levels of 5α-reductase-3 and 5α-reductase-1 were similar in AS-CWR22 and CR-CWR22 xenografts (Fig. 4A, upper panel). In contrast, 5α-reductase-2 mRNA in CR-CWR22 xenografts was double that of AS-CWR22 xenografts. Similarly, gene expression of HSD17β3 and AKR1C3 were 3.4-fold and 10.6-fold higher, respectively, in CR-CWR22 xenografts than in AS-CWR22 xenografts. However, on Day 20 after castration, xenograft transcript levels of 5α-reductase-1, -2, -3, HSD17β3, and AKR1C3 were similar in AS-CWR22 and CR-CWR22 tumors (data not shown).

AS-CWR22 and CR-CWR22 xenograft lysates demonstrated persistent formation of 5α-ASD and DHT from androstenedione after preincubation with dutasteride. 5α-ASD can be converted to DHT by 17β-oxidoreductase enzymes [25,26]. The total amount of DHT and 5α-ASD biosynthesized in control lystate incubations with 1.0 μM androstenedione were 2,100 pmol/mg protein/hr in AS-CWR22 and 1,800 pmol/mg protein/hr in CR-CWR22 (Fig. 4A, lower panel). AS-CWR22 lysates preincubated with 7.0 nM dutasteride demonstrated a ~80% decrease in total 5α-ASD and DHT biosynthesis. Similarly, CR-CWR22 tumor lysates preincubated with 7.0 nM dutasteride showed a ~80% decrease 5α-ASD and DHT biosynthesis. Dutasteride substantially inhibited 5α-reductase activity in both AS-CWR22 and CR-CWR22 lysates by irreversibly binding at the active site of 5α-reductase-1 and -2. The persistent levels of 5α-ASD observed in the presence of dutasteride suggests that dutasteride is not a mechanism-based inhibitor of 5α-reductase-3 and that high levels of androstenedione are 5α-reduced by endogenous 5α-reductase-3 [26,27].

Recombinant 5α-reductase-3 expressed in bacteria converted progesterone to DHP. Previous reports support that progesterone inhibited 5α-reductase-1 and -2 activity in human tissue lysates [28], which suggests that 5α-reductase-3 (low sequence identity with 5α-reductase-1) may be the principal mechanism for 5α-reduction of progesterone. Furthermore, progesterone levels increased in urine from men treated with abiraterone acetate alone or abiraterone acetate and dexamethasone [29] supporting increased systemic progesterone levels. Hence, 5α-reductase-3 mediated conversion of radiolabeled progesterone to androgen precursors and ultimately to DHT was qualitatively characterized using lysates of AS- and 40-, 80-, and120-day postcastrate and CR-CWR22 xenografts (Fig. 4B). The presence of DHT as a metabolite supports CYP17A1 and 5α-reductase-3 enzyme activity in all lysates. AS-CWR22 lysates demonstrated low conversion of progesterone to DHT (0.15%), androstenedione (1.64%), and testosterone (0.08%). In contrast, the CWR22 lysates at longer times postcastration demonstrated progressively increased conversion of progesterone to DHT, which ranged from 0.5% (40 day castrate) to 1.8% in CR-CWR22. In postcastration xenografts, androstenedione was the most prevalent metabolite, and ranged from 4% (120 days castrate CWR22) to 18% (CR-CWR22) of total radiolabeled progesterone. The percent of radiolabel present in testosterone was low, and similar (~0.3%) in lysates from 40-, 80-, 120-day castrate CWR22 tumors as were testosterone levels in AS-CWR22 and CR-CWR22 tumors. The low testosterone (0.07%) and high androstenedione

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levels (18%), and levels, observed in CR-CWR22 lysates incubated with progesterone suggests the potential for two biosynthetic pathways for DHT formation: backdoor biosynthesis of DHT from progesterone [26,30] and formation of DHT after catabolic 5α-reduction of androstenedione to 5α-ASD followed by further reduction to DHT [26].

5α-Reductase Gene Expression and Enzyme Activity in Clinical Specimens

The total level of mRNA expression of 5α-reductase-1, -2, and -3 in AS-BP tissues was used as the standard to evaluate the relative 5α-reducing capabilities of human CaP. Relative to the total 5α-reducing mRNA available in AS-BP, the mean levels of 5α-reductase-1 mRNA decreased 46% in AS-CaP and 81% in CR-CaP tissue (Fig. 5A). Similarly, expression of 5α-reductase-2 mRNA was decreased 96% in AS-CaP and 99.8% in CR-CaP tissues. In contrast, expression of 5α-reductase-3 mRNA was decreased 60% in AS-CaP, but only 53% in CR-CaP tissues.

In AS-BP and AS-CaP tissues, testosterone was 5α-reduced directly to DHT (range 3.80–0.04 nM), or oxidized to androstenedione, which then was 5α-reduced to 5α-ASD (range 4.0–1.01 nM) that was metabolized further to androsterone (range 0.46–0.02 nM) (Fig. 5B, upper and middle panels). Pre-incubation of AS-BP or AS-CaP with dutasteride resulted in a dose-dependent inhibition of the 5α-reduction of testosterone to DHT. In contrast, dutasteride's effects on the metabolic conversion of testosterone to 5α-ASD and androsterone levels were negligible in these specimens.

Lysates of AS-BP and AS-CaP tissue, when incubated with androstenedione were capable of 5α-reduction of androstenedione to 5α-ASD (Fig. 5C, upper and middle panels). Dutasteride did not inhibit appreciably the 5α-reduction of androstenedione to 5α-ASD under the incubation conditions established for the tissue lysates. Essentially no androsterone was apparent in these incubations, which suggests that aqueous soluble conjugates were not recovered. DHT was produced at low levels by both AS-BP or AS-CaP lysates, which supports the hypothesis that HSD17β isoforms necessary to reduce androstenedione to testosterone were not present or active in the lysates.

The metabolite profiles of testosterone or androstenedione obtained from lysates of a limited set of CR-CaP tissue specimens were markedly different (Fig. 5B and Fig. 5C lower panels). Testosterone was converted exclusively to DHT whereas 5α-ASD and androsterone were not observed. Interestingly, DHT levels persisted and were quantifiable at all dutasteride concentrations (Fig. 5B lower panel). In contrast, androstenedione was metabolized to 5α-ASD and DHT, and androsterone was not observed. The 5α-ASD and DHT levels were not substantially altered from controls in the presence of dutasteride (Fig. 5C, lower panel). The 5α-ASD levels were consistently high (15 nM) and the DHT persisted at low levels (<0.4 nM).

DISCUSSION

The presence of a third 5α-reductase was suggested by the discovery of a new 5α-reductase-like gene (SRD5A2L, www.genecards.org); the unexpected detection of DHT in sera of intact male 5α-reductase-1 and -2 knockout mice [16]; the inability of a dual 5α-reductase

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inhibitor, dutasteride, to eliminate serum [5] and tissue levels of DHT [20] in AS-BP; and the limited benefit of dutasteride in treatment of men with CR-CaP [12]. The observation by Uemura et al. [31] that recombinant 5α-reductase-3 converted testosterone to DHT, but mutated 5α-reductase-3 was inactive, validated the identity of a new 5α-reductase. Our data confirm that the mammalian and bacterial expressed recombinant 5α-reductase-3 enzyme5α-reduced the 3-keto-Δ4-ene steroids, testosterone, androstenedione, and progesterone. Although over-expression of 5α-reductase-3 proved difficult because of its hydrophobic amino acid content, the adenovirus, and 6-His-tag-thiofusion expression vectors produced sufficient quantity of active enzyme for in vitro enzyme assays.

Previous studies determined that recombinant 5α-reductase-1 and -2 enzymes have similar testosterone K_m values (~1 μM [18]) and dutasteride inhibition values (~7.0 nM [2]). At 1.0 μM testosterone, recombinant 5α-reductase-3 demonstrated stable activity (~40 pmol/mg protein/hr) at both acidic and basic pH, which is similar to the pH profile of 5α-reductase-1 [18,19]. 5α-Reductase-3 exhibited increased specific activity at pH 6.5which confirmed the critical catalytic role of the conserved histidine (H296, pKa ~6–7) in the conversion of testosterone to DHT established by Uemura et al. [31]. In the mammalian expression system, the rate of testosterone conversion to DHT by recombinant 5α-reductase-3 was less efficient than recombinant 5α-reductase-1 and -2 [18]. Dutasteride (100 or 150nM) partially inhibited both endogenous and recombinant 5α-reductase-3 reduction of testosterone to DHT at neutral pH. The percent inhibition of DHT biosynthesis using CHO-K1 cell lysates pre-incubated with 100 or 150 nM dutasteride was similar.

These results suggest that 5α-reductase-3 (20% amino acid identity to 5α-reductase-1) is not an isozyme of the same family as 5α-reductase-1 and -2. Similarly, 100 and 150 nM abiraterone minimally inhibited 5α-reductase-3. Two recent reports established that 5α-reductase-3 is classified in a unique gene family separate from 5α-reductase-1 and -2 [32] and is required for reducing polyprenol to dolichol, a precursor used in N-glycosylation of proteins [33]. The 5α-reductase-3 homologue in tissue homogenates from intact male mice lacking the 5α-reductase-1 and -2 genes also did not convert testosterone to DHT at neutral or acidic pH [16]). However, our data demonstrate that recombinant 5α-reductase-3 incubated with a high testosterone concentration produced DHT in both the presence and absence of dutasteride. Taken together, these data suggest that 5α-reductase-3 is not effectively inhibited by dutasteride and that testosterone at high concentrations may be an alternative substrate.

The observation that semi-purified over-expressed 5α-reductase-3 from bacteria 5α-reduced testosterone, androstenedione, and progesterone led to evaluation of DHT formation of these androgen precursors using the CWR22 CaP model. CWR22 xenograft lyates metabolized androstenedione or progesterone to DHT and preincubation with low concentrations of dutasteride reduced but did not eliminate androstenedione conversion to 5α-ASD or DHT. The biosynthesis of 5α-ASD in AS-CWR22 and CR-CWR22 incubations in the presence of dutasteride supported the metabolism of androstenedione to DHT via sequential 5α-reductase-3 and AKR1C3 or HSD17β3 enzyme activity (Fig. 6). A report by Chang et al. [26] described this novel metabolic pathway to DHT via 5α-reductase conversion of androstenedione to 5α-ASD prior to DHT formation in DU145 cells. These data support

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efficient reduction of androstenedione by 5α-reductase-3 in CWR22 xenografts with depleted 5α-reductase-1 and -2 activity.

CWR22 tumor metabolism of progesterone to DHT further supports the hypothesis that DHT biosynthesis does not require testosterone. All castrate CWR22 tumor incubations formed androstenedione although testosterone levels were negligible. This androgen profile supported CYP17A1 metabolism of progesterone to androstenedione and subsequently 5α-ASD and DHT biosynthesis by 5α-reductase-3 and AKR1C3 or HSD17β3, respectively. However, the low percentage conversion of progesterone to androstenedione, except in CR-CWR22 tumors, supports the presence of an alternative pathway for DHT biosynthesis from progesterone (Fig. 6). 5α-Reductase-3 formation of DHP may select for DHT biosynthesis by the “backdoor pathway” [30,34]. In the presence of dutasteride, DHP formed by uninhibited 5α-reductase-3 can be further metabolized by P450 17α-hydroxylase/17,20-lyaseto-precursors of androsterone [34] and ultimately DHT by sequential HSD17β/3α-HSD oxidoreductase activity [35]. In corroboration of this pathway in the CWR22 model is the observation that recurrent LNCaP tumors metabolized progesterone to DHT and demonstrated increased AKR1C2, CYP17A1, and AKR1C3 transcript levels [27]. Further, Montgomery et al. [36] demonstrated increased CYP17A1 and AKR1C3 transcript levels in CaP bone metastases than in primary CaP from human warm autopsy specimens of CR-CaP. 5α-ASD and DHP may be important intraprostatic 5α-reduced metabolite biomarkers in CR-CaP and in clinical chemoprevention trials. Thus, intracrine DHT biosynthesis may occur via metabolism of an adrenal androgen, androstenedione, through 5α-ASD that accumulates to favor DHT formation by AKR1C3 [26]. Alternatively, DHT can be biosynthesized via the “backdoor pathway” metabolism of progesterone to DHP [30,37].

The observation that 5α-reductase-3 5α-reduced alternate steroid substrates, testosterone, androstenedione and progesterone, and evidence of residual dolichol measured in 5α-reductase-3 mutant cells [33], suggest that 5α-reductase-1, -2, and -3 enzymes are catalytically promiscuous [38]. 5α-Reductase-1, -2, and -3 NADPH dependent catalysis of secondary substrates confirms the use of a common catalytic mechanism in the reduction of polyprenol terminal vinyl alcohol and Δ4-ene steroids. The kinetic mechanism for steroid 5α-reduction by 5α-reductase-1 and -2 and the intermediate state formed in the active site have been defined [39–41]. The enolate intermediate formed by 5α-reductase-1 and -2 prior to NADPH reduction is analogous to the polyprenol terminal vinyl alcohol (Fig. 7). 5α-reductase-1 and -2 may bind the vinyl alcohol functional group as a intermediate mimic and thus reducing the double bond, but at a lower rate than 5α-reductase-3. Similarly, 5α-reductase-3 metabolism of testosterone, androstenedione, and progesterone may occur via binding of the steroid enolate to promote Δ4-ene 5α-reduction. The different percentage of inhibition observed with dutasteride versus abiraterone is supported by formation of dutasteride enolate intermediate at the active site of 5α-reductase-3, whereas abiraterone cannot form an enolate intermediate.

Clinical relevance for a new 5α-reductase was suggested using a limited number of clinical specimens and the CWR-22 model to provide coordinate mRNA and enzymatic analyses. Titus et al. [19] and others [37,42,43] reported previously that 5α-reducing capacity shifted from 5α-reductase-2 in AS-BP to 5α-reductase-1 in AS-CaP and CR-CaP. 5α-Reductase-3

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gene transcript levels were similar in AS-CaP and CR-CaP. The relative relationship of 5α-reductase enzyme gene expression in CaP were 5α-reductase-3 >5α-reductase-1 >5α-reductase-2 in AS-CaP and 5α-reductase-3 ≫ 5α-reductase-1 >5α-reductase-2 in CR-CaP. Uemura et al. [31] also demonstrated up-regulation of 5α-reductase-3 mRNA levels in CR-CaP specimens. As with previously observed protein levels [19], our AS-CaP 5α-reductase-1 and 5α-reductase-2 mRNA levels were similar but maintained at higher levels than either 5α-reductase-1 or 5α-reductase-2 mRNA levels in CR-CaP.

The in vitro incubations using tumor lysates of AS-BP, AS-CaP, and CR-CaP specimens with higher concentrations of dutasteride than can be achieved clinically [20] also demonstrated persistent biosynthesis of DHT, 5α-ASD, and androsterone. The DHT measured in AS-BP, AS-CaP, and CR-CaP incubations at high concentrations of testosterone and dutasteride is most likely from uninhibited 5α-reductase-3 metabolism of testosterone because of decreased 5α-reductase-2 expression in CR-CaP and dutasteride's inhibition of 5α-reductase-1. The observed 5α-ASD in AS-BP and AS-CaP may be from 5α-reductase-3 reduction of androstenedione after HSD17β2 oxidation of testosterone [44]. The expression of HSD17β2 is reduced in advanced CaP [44] as confirmed by the absence of 5α-ASD in our CR-CaP incubations. The low DHT levels relative to the 5α-ASD level observed in AS-BP and AS-CaP at high dutasteride concentration may be a result of AKR1C3's slow conversion of 5α-ASD to DHT in the clinical specimens. The loss of androsterone in AS-CaP and CR-CaP could result from phase 2 conjugation of DHT metabolites by UDP-glucuronosyl transferase 2B15 enzyme activity [45].

The predominance of 5α-ASD in AS-BP, AS-CaP, and CR-CaP specimens incubated with androstenedi-one suggest that substantial 5α-reductase-3 activity precedes HSD17β's reduction to DHT in the presence of dutasteride. In AS-BP and AS-CaP incubations, androstenedione is converted to 5α-ASD followed by HSD17β reduction to DHT. The decrease inactive AKR1C3 limited the formation of DHT in all clinical specimen incubations. However, androstenedione appears to be the preferred substrate for 5α-reduction in CR-CaP incubations in both the presence or absence of dutasteride. The data presented in Fig. 5 provide evidence that 5α-reductase-3 enzymatic activity in the tissue lysates is not inhibited strongly by dutasteride in these in vitro incubation conditions, which include supra-physiological concentrations of substrate. These results also suggest that 5α-reductase-3 may play an important role in metabolizing the Δ4-3-keto steroids testosterone, androstenedione [19,26] and progesterone to DHT and 5α-reduced precursors of DHT in the presence of dutasteride via multiple biosynthetic pathways activated during the progression of AS-BP to advanced CaP.

CONCLUSIONS

The relative contributions of 5α-reducatase-1, -2, and -3 to the 5α-reducing capacity in AS-CaP and CR-CaP are difficult to evaluate. 5α-reductase enzyme activity assessed under artificial conditions of tissue lysates are influenced by differences in pH [18,19], availability of NADPH and tissue heterogeneity. However, the data suggest that 5α-reductase-3 activity may replace inhibited [7,12,46,47] and decreased 5α-reductase-2 [19,43] activity in AS-CaP and CR-CaP. 5α-Reductase-3 protein expression in these tissues [17] can sustain intracrine

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metabolism of testosterone, androstenedione[19,48,49], and progesterone to DHT. Furthermore, the 5α-reduced intermediates, 5α-ASD and DHP, can be metabolized to DHT [34,37,45,50,51], which suggests an important role for 5α-reductase-3 in the lethal phenotype of CaP.

Acknowledgments

We thank Mei-Heng Mar and Carol Wrzosek for their technical assistance. This work was supported by Grant Sponsor: The National Cancer Institute at the National Institutes of Health, (P01-CA77739 to J.L.M.); Grant Sponsor: the Department of Defense Prostate Cancer Research Program Grant (W81XWH-10-1-0273 to M.A.T.); Grant Sponsor: National Cancer Institute Cancer Center Support Grants to Roswell Park Cancer Institute (CA016156), the University of North Carolina (CA034026), and The University of Texas MD Anderson Cancer Center (CA016672).

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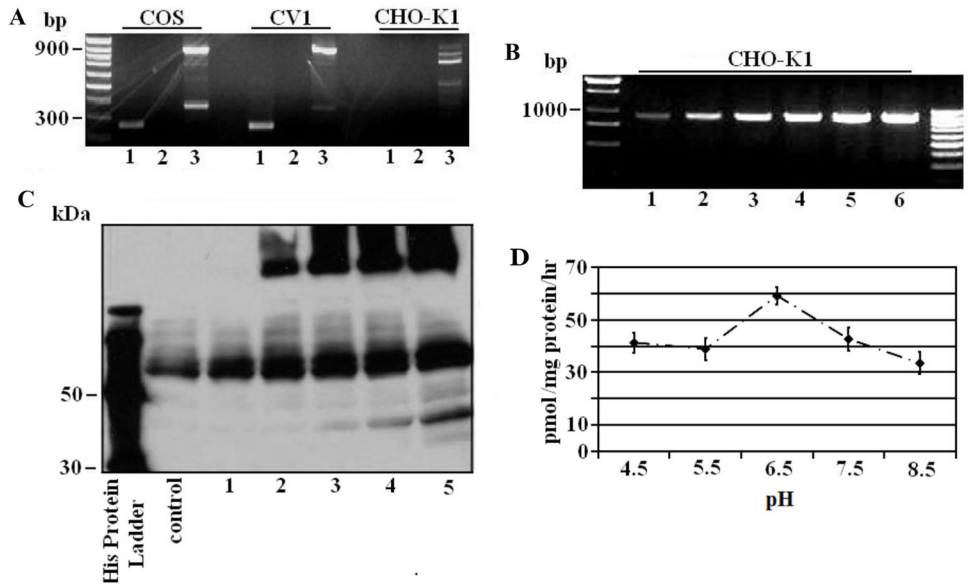

Fig. 1.

5α-Reductase enzyme mRNA expression, protein expression, and enzymatic and inhibition assays using CHO-K1cells. **A**: 5α-Reductase-1 (lane1, 270 bp), 5α-reductase-2 (lane 2,155 bp, not observed), and 5α-reductase-3 (lane 3,954 bp) endogenous gene expression in COS, CV-1, and CHO-K1cells (representative of three replicates of each of three independent experiments).**B**: 5α-Reductase-3 gene expression in uninfected (lane 1) and adenovirus infected CHO-K1 cells (lanes 2–6) at 48 hr. 5α-Reductase-3 adenovirus infectious units (ifu) were increased from 1.3×10^5 (lane 2) to 5.2×10^7 (lane 6) (representative of three replicates of each of three independent experiments). **C**: 5α-Reductase-3 immunoblot using adenovirus infected CHO-K1cell protein lysate (1.3×10^6 ifu, lane1to1.3×10^8 ifu, lane 5). 5α-Reductase-3 protein (33 kDa) was detected with anti-6X-His antibody. **D**: 5α-Reductase-3 enzyme activity (DHT and 5α-ASD formedin pmol/mg protein/hr from 1.0 μM testosterone) determined over pH range 4.5–8.5 using adenovirus (1.3×10^8 ifu) infected CHO-K1 protein lysate (three replicates at each pH and representative of three independent experiments).

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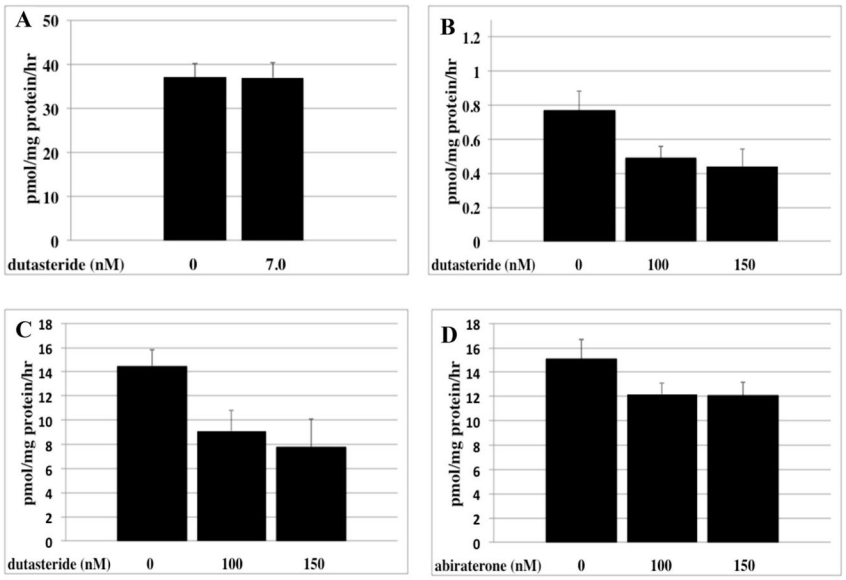

Fig. 2.

Inhibition study of expressed or endogenous 5α-reductase-3 enzyme in the absence and presence of 7.0,100, and150 nM dutasteride (bispecific 5α-reductase-1and -2 inhibitor), and100 and150 nM abiraterone at pH 7.4 using1.0 μM testosterone as substrate. **A**: CHO-K1protein lysate from high adenovirus infected cells treated with 7.0 nM dutasteride (the IC_{50} for inhibition of 5α-reductase-1 and -2) demonstrated no inhibition. **B**: Uninfected control CHO-K1protein lysate treatedwith100 and150 nM dutasteride showed ~40% inhibition of endogenous 5α-reductase-3.**C**: CHO-K1protein lysate from low adenovirus infected cells treated with100 and150 nM dutasteride showed ~45% inhibition of expressed 5α-reductase-3, and (**D**) ~20% inhibition using100 and150 nM abiraterone (three replicates at pH 7.4 and representative of two independent experiments).

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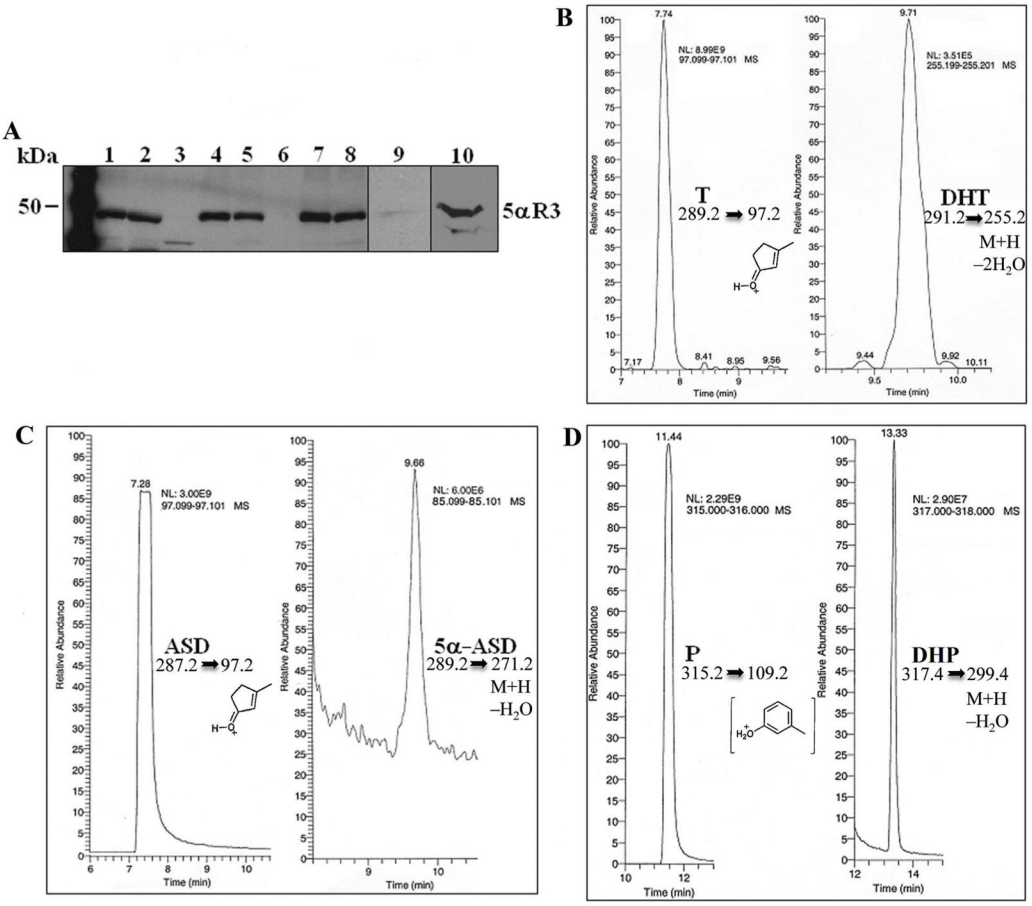

Fig. 3.

Recombinant 5α-reductase-3 (5αR3)Thiofusion protein (37 kDa)immunoblot analysis using anti-6X-His and anti-Thio antibodies. (**A**) Lanes1and 2: bacterial lysates using two preparations; lane 3: lysate from un induced bacteria; lanes 4 and 5: nickel-bead-semipurified 5α-reductase-3; lane 6: nickel-bead purification from un induced bacteria; lanes 7 and 8: nickel-bead-semipurified 5α-reductase-3 after a two volume column wash; lane 9: 5α-reductase-3 detected in bacterial lysate using anti-Thio antibody; lane10: 5α-reductase-3 detected in bacterial pellet using polyclonal antibody. Immunoblots are representative of two independent experiments. **B** –**D**: Semipurified expressed 5α-reductase-3 enzyme (50 μg) substrate specificity using 0.5 μM testosterone (T), androstenedione (ASD), or progesterone (P).Mass spectrometry chromatograms demonstrated 5α-reductase-3 conversion of T to DHT (B), ASD to 5α-ASD (C) and P to 5α-pregnan-3-20-dione (DHP, D).The parent and fragment ion pairs monitored were 289.2 to 97.2 for T, 291.2 to 255.2 for DHT, 287.2 to 97.2 for ASD, 289.2 to 271.2 for 5α-ASD, 315.2 to109.2 for P, and 317.4 to 299.4 for DHP. Product ion structures are shown for T, ASD, and P. Product ions for DHT, 5α-ASD, and DHP are describe as loss of water from molecular ion (M+H). Each chromatogram is the best from two replicates of two separate protein expression preparations.

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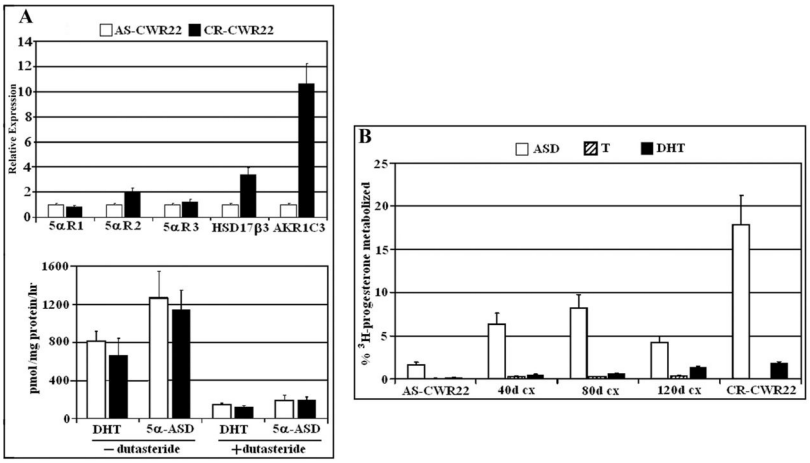

Fig. 4.

Endogenous 5α-reductase-3 gene expression and enzyme activity using CWR22 xenografts from intact and castrated mouse hosts. (**A**, upper panel) The fold increase in 5α-reductase (5αR)-1, -2, -3,17β-hydroxysteroiddehydrogenase-3 (HSD17β3), and aldo-keto reductase 1C3 (AKR1C3) transcript levels in CWR22 xenografts before castration (AS-CWR22, white) and recurrent xenograft after castration (CR-CWR22, black). (A, lower panel) Dihydrotestosterone (DHT) and 5α-androstan-3,17-dione (5α-ASD) formation in AS-CWR22 (black) and CR-CWR22 (black) xenograft lysates incubated at pH 7.4 in1.0 μM androstenedione with or without 7.0 nM dutasteride. **B**: The percentage of[^{3}H] progesterone metabolized to androstenedione (ASD), testosterone (T), and dihydrotestosterone (DHT), usingAS-CWR22, castrate (cx) 40, 80,120-days (d), and CR-CWR22 xenograft lysates at pH 7.4.Data are three replicates of experiments performed on each of three xenograft samples).

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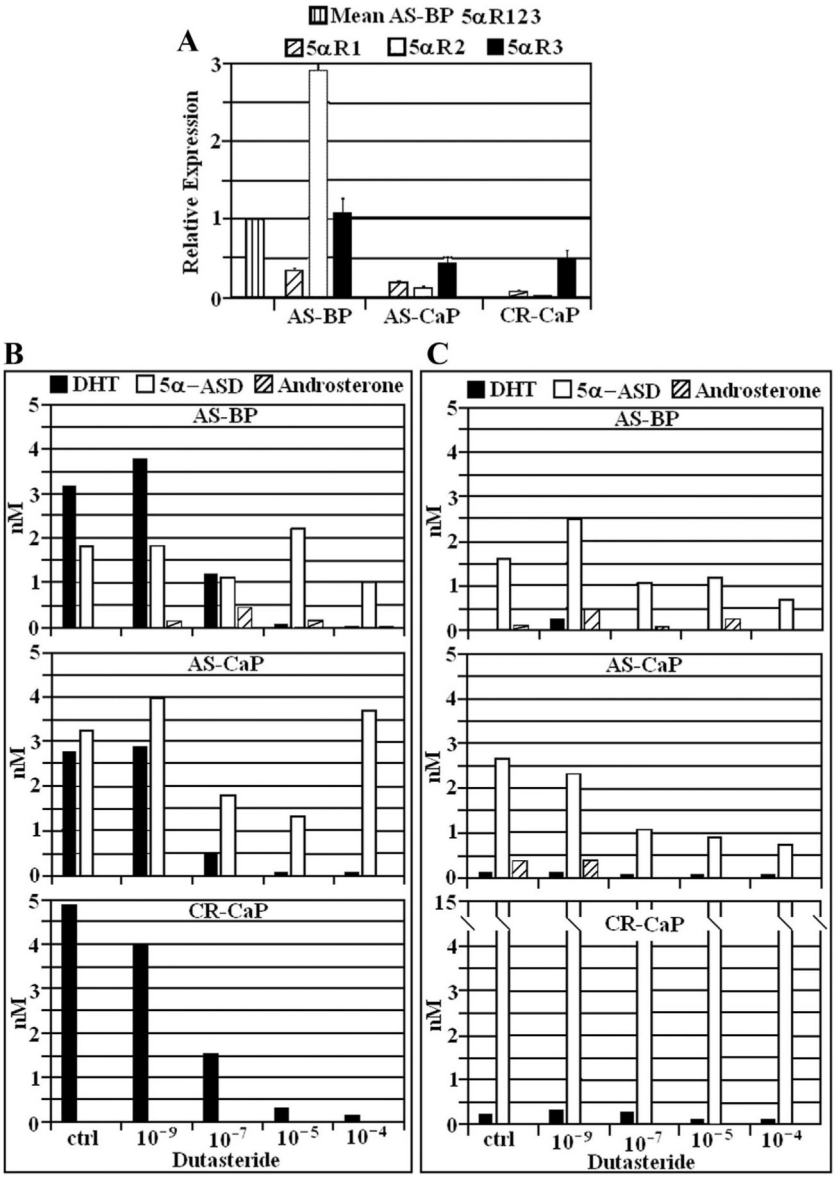

Fig. 5.

5α-Reductase-1, -2, and -3 mRNA levels by qRT-PCR and 5α-reductase enzyme activity in clinical prostate cancer (CaP) specimens. **A**: Transcript levels of 5α-reductase-1, -2, and -3 in androgen-stimulated CaP (AS-CaP) and CRCaP (CR-CaP) relative to androgen-stimulated benign prostate (AS-BP).Three specimens from each tissue type were used and data are reported as mean ± SD. **B** and **C**: Average levels of 5α-reduced androgens dihydrotestosterone (DHT), 5α-androstan-3,17-dione (5α-ASD), and 5α-androstan-3α-ol-17-one (androsterone) formed in incubations of clinical prostate specimens. 5α-reductase enzyme activity in AS-BP (n = 3), AS-CaP (n = 3), and CR-CaP (n = 3) in the presence of dutasteride (10^{-9} to10^{-4} M or1.0 nM to100 μM and should be compared with100 nM, the maximum tissue concentration achievable clinically (20)) using (B) 1.0 μM testosterone or (C) 1.0 μM androstenedione as substrate. Data are reported as the means of two replicates of tissue combined by type.

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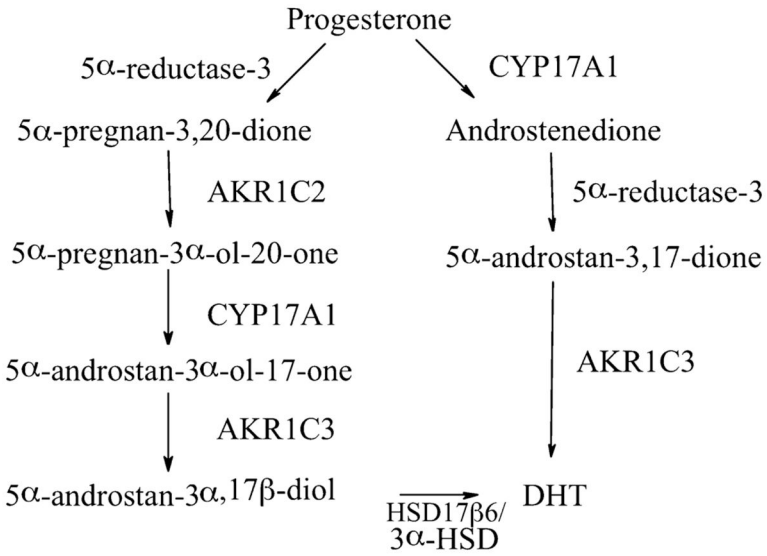

Fig. 6.

A schematic outline of two sequential steroidogenic pathways for the biosynthesis of dihydrotestosterone (DHT) in the presence of dutasteride. The backdoor pathway integrating 5α-reductase-3 metabolism of progesterone to 5α-pregnan-3-20-dione (DHP), and a second pathway that converts progesterone to androstenendione followed by androstenedione conversion to 5α-androstan-3-20-dione (5α-ASD) by 5α-reductase-3. The steroidogenic enzymes are 5α-reductase-3, aldo-keto reductase family1C (AKR1C), cytochrome P450 17α-hydroxylase17/20 lyase (CYP17A1), 17β-hydroxysteroid dehydrogenase (HSD17β6), and 3α-hydroxysteroid dehydrogenase (3α-HSD).

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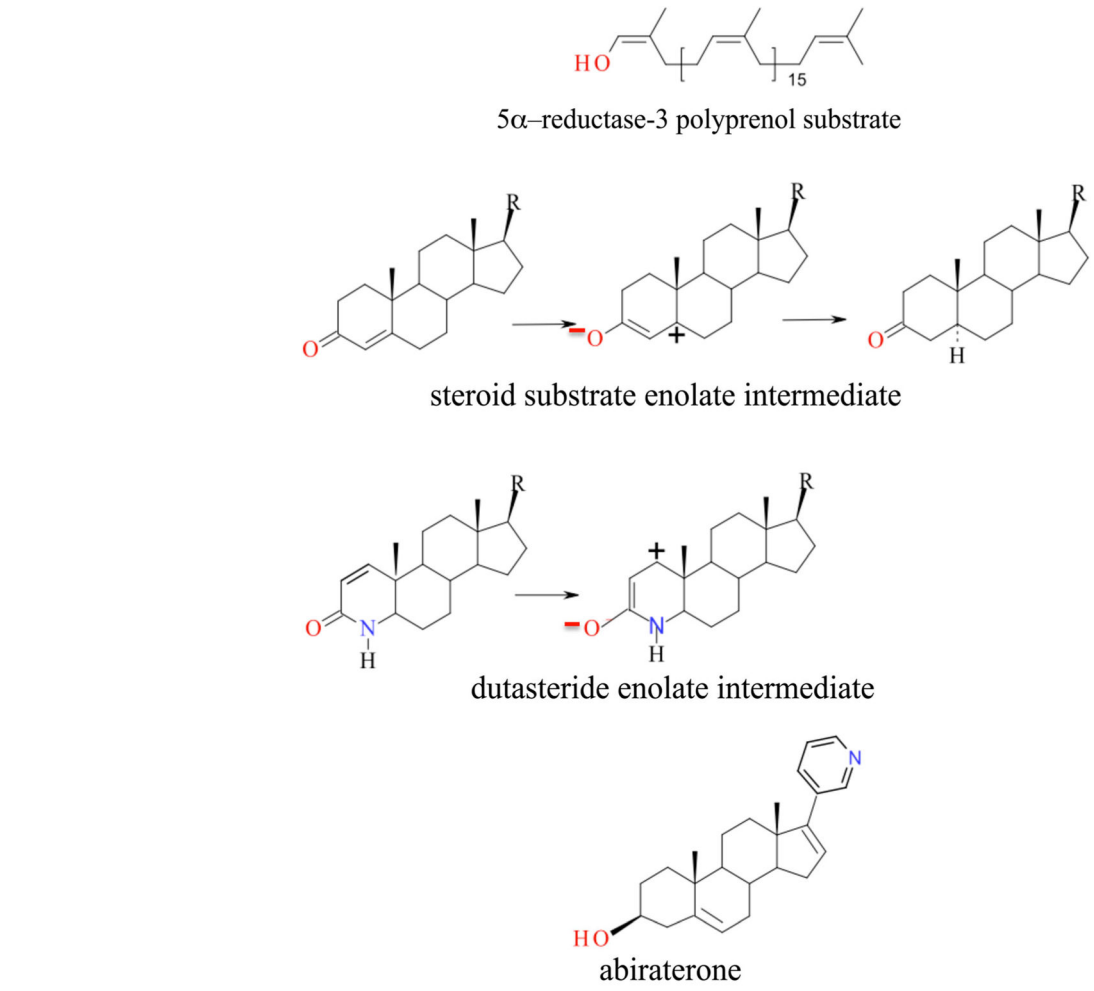

Fig. 7.

Chemical structures of the primary and alternative 5α-reductase-3 substrates and clinical steroidogenic inhibitors. The primary substrate, polyprenol, shows the terminal vinyl alcohol functional group (red) that is reduced to saturated alcohol by 5α-reductase-3 enzyme. The alternative steroid substrate stestosterone (R = hydroxyl), androstenedione (R = ketone) and progesterone (R = methylketone) and the enolate intermediate stabilized by 5α-reductase-1and -2 catalytic mechanism that resemble the terminal vinyl alcohol of polyprenol. The enolate intermediate of dutasteride resembles both the steroid enolate and polyprenol terminal vinyl alcohol. Abiraterone's structure is similar to that of the reduced alcohol product of polyprenol substrate.