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Inhibition of the Activity of 'Basic' 5α-Reductase (Type 1) Detected in DU 145 Cells and Expressed in Insect Cells

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The purpose of this study was 2-fold: (1) to identify the 5α-reductase (5α-R) isozyme(s) present in DU 145 cells, a human cell-line of low androgen sensitivity derived from a cerebral metastasis of an epithelial prostate cancer; and (2) to compare the inhibitory potencies of three compounds on the 'basic' 5α-R isozyme expressed in a baculovirus-directed insect cell system. Conversion of testosterone (T) into 5α-dihydrotestosterone (DHT) in DU 145 cells was measured by HPLC coupled to a Flo-one HP radioactivity detector. DU 145 cells exhibited 5α-R activity (21 pmol DHT/min/mg protein) at pH 7.4 which disappeared at pH 5.5 suggesting that, of the two genomically distinct human isozymes identified so far, type 1 5α-R is expressed in DU 145 cells. This was confirmed by at least two observations: first, 5α-R activity in DU 145 cells was inhibited with much higher potency by 4-MA than by finasteride which is known to be a very poor competitor of the 'basic' enzyme (IC_{50}s = 2.8 ± 0.2 and 264 ± 55 nM, respectively). Second, only the type 1 5α-R cDNA and not type 2 5α-R cDNA hybridized with DU 145 RNA. A high potency differential was also recorded for the inhibition of 'basic' type 1 5α-R expressed in a baculovirus-directed-insect cell system by these two compounds, 4-MA being considerably more active than finasteride (K_i = 8.4 ± 2.3 and 330 ± 9 nM, respectively). This inhibition was competitive. On the other hand, inhibition by an *n*-hexane lipid/sterol extract of *Serenoa repens* (LSESr) was non-competitive and, when expressed in terms of recommended therapeutic doses, was 3-fold greater for LSESr than for finasteride. These studies suggest that LSESr might exert a regulatory inhibitory activity due to its specific lipid/sterol composition.

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INTRODUCTION

The enzyme 5α-reductase (5α-R), present in the prostate, seminal vesicles, sebaceous glands, brain and other tissues, reduces steroids at the 5 position using NADPH as a cofactor. In the prostate, testosterone (T) is directly converted into 5α-dihydrotestosterone (5α-DHT), one of several factors regulating normal and pathological prostate cell growth, and indirectly into other 5α-reduced metabolites, 5α-androstanedione and 5α-androstanediols [1, 2]. It has been long thought that there may be at least two 5α-R isoforms [3–5] and this has been recently confirmed by cloning and expressing two isozymes, type 1 [6] and type 2 [7, 8], from human prostate [9]. The type 1 isozyme has a neutral-basic pH optimum (7.0–8.0) and the type 2 isozyme an acidic pH optimum (5.0).

The first aim of the present study was to characterize the enzyme isoform present in the DU 145 cell-line. This cell-line is derived from a brain metastasis of an epithelial human prostate carcinoma [10]. Its growth is generally, but not always [11], considered to be insensitive to androgen and its androgen receptor (AR) status to be negative. Nevertheless, these cells contain

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Abbreviations: BPH: benign prostate hypertrophy; AR: androgen receptor; 5α-R: steroid 5α-reductase (3-oxo-5α-steroid: $NADP^+$ 4-en-oxido-reductase, EC 1.3.1.22); T: testosterone (17β-hydroxy-4-androsten-3-one); 5α-DHT: 5α-dihydrotestosterone (17β-hydroxy-5α-androstan-3-one); 4-MA: 17β-(*N*, *N*,-diethyl)-carbamoyl-4-aza-5α-androstan-3-one; finasteride *N*-(2-methyl (2-propyl)-3-oxo-4-aza-5α-androst-1-ene-17β-carboxamide; LSESr: lipid/sterol extract from *Serenoa repens* fruit (*Sabal fructus*); AcNPV: *Autographa californica* nuclear polyhedrosis virus.

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5α-R. Recently, they have been used to compare the action of several 4-azasteroids [12], designed principally with the treatment of benign prostate hypertrophy (BPH) in mind, even though their epithelial origin, malignant nature and AR-negativity detract somewhat from their relevance as a model for BPH. Contrary to the conclusion of this study [12], our results will show that DU 145 cells only express the 'basic' type 1 and not 'acidic' type 2 isozyme.

We have recently produced the 'basic' type 1 human enzyme in an eukaryotic expression system, the baculovirus-directed insect cell system, which provides high yields of biologically active and stable protein [13]. The second aim of our study was therefore to compare inhibition of the type 1 enzyme by different agents, namely, two aza-steroids [4-MA and finasteride (MK-906)] [14–18] and the *n*-hexane extract of the fruit of the dwarf american palm *Serenoa repens* (LSESr). LSESr is a mixture of free fatty acid and sterol components which inhibits 5α-R activity and AR binding in rat prostate tissue [19, 20] and in cultured human foreskin fibroblasts [21]. The present study establishes that, unlike the 4-azasteroids, LSESr is a non-competitive inhibitor of type 1 5α-R indicating that it belongs to a new class of inhibitors with a regulatory mode of action. Recent investigations have suggested that overspecific and stringent 5α-R inhibition in prostate may be clinically counterproductive because of the formation of high tissue concentrations of biologically active T in spite of much reduced 5α-DHT levels [22, 23]. It is known that T has an affinity for AR that is about 80% of that of 5α-DHT [24] and that high concentrations can exert a growth-promoting action like 5α-DHT [25, 26].

MATERIALS AND METHODS

Chemicals

[1,2,6,7-^{3}H]Testosterone (97 Ci/mmol) was purchased from Amersham (UK). Diethyl ether (Prolabo, France) was of analytical grade. Methanol and tetrahydrofuran (Prolabo) were glass distilled in our laboratory. Deionized water was triple glass distilled. Unlabeled steroids were purchased from Sigma (France) and NADPH (621706) from Boehringer (Mannheim, Germany). 4-MA and finasteride were synthesized at the Research Laboratories of Farmitalia Carlo Erba (Milan, Italy). LSESr (lipid/sterol extract from *Serenoa repens* fruit, batch HC 2513) was from Pierre Fabre Médicament (Castres, France).

Cell cultures

The human prostatic carcinoma cell-line DU 145 (ATCC, HTB 81) [10] was maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2 in 75 cm^2 cell culture flasks (Corning, Staffordshire, England). Cells were routinely cultured in RPMI-1640 medium with 10% fetal calf serum, 100 U/ml penicillin, 100 mg/ml streptomycin, 1 mM sodium pyruvate, 2 mM L-glutamine (all from Gibco, Middlesex, England). They were trypsinized and replated into fresh medium every 5 days. Assays were performed on cells between passages 10–20.

Spodoptera frugiperda (Sf9) insect cells (ATCC, CRL 1711) were maintained at 27°C in Grace's insect medium supplemented with 10% fetal calf serum (FCS), 3.3 g/l lactalbumin hydrolysate, 3.3 g/l yeastolate (all from Gibco/BRL), 50 mg/ml gentamycin sulfate and 2.5 mg/ml amphotericin B (Sigma).

Assay of 5α-R activity in DU 145 cells

DU 145 cells (ca. 2×10^5) were plated in 35 mm wells of 6-well cluster plates (Corning) in RPMI-1640 containing 1% heat-inactivated calf serum depleted of steroids by dextran-coated charcoal (DCC) treatment, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, and 1 nM sodium pyruvate. After 3 days, the medium was replaced by 1 ml fresh medium containing 0.1 μM T, 0.6×10^6 cpm [^{3}H]T and various concentrations of inhibitor. After incubation for 1 to 6 h, the medium was removed and stored at −20°C. The cells were harvested by trypsination and counted in a Coulter counter (Coultronics, France).

The pH specificity of 5α-R activity was determined as follows. Subconfluent cultured DU 145 cells in six 150 cm^2 flasks were washed twice with phosphate-buffered saline. After centrifugation at 1000 *g* for 10 min, the cell pellet was suspended in 50 mM Tris–EDTA buffer of different pH strengths and sonicated at 4°C for 30 s at 2/3rds of maximal power (Ultrasons Annemasse, France). The BCA protein assay reagent (Pierce, Rockford, IL, U.S.A.) was used to determine cytosolic protein concentration. A 50 μl aliquot of cell homogenate containing 2 mg/ml protein was then added to the reaction mixture (250 μl) containing: 1 μM T, 0.6×10^6 cpm [^{3}H]T and 5 mM NADPH. After 10 min at 37°C, the reaction was stopped by immersion of the tube in liquid nitrogen. The sample was stored at −20°C until steroid assay.

Analysis of T metabolism

Radioactive T and DHT were measured as described previously [27]. They were extracted from the incubation medium twice with 3 ml diethyl ether. The solvent was evaporated and the residue taken up in 200 μl of methanol–tetrahydrofuran–water (40:13:47, by vol). A 100 μl sample was injected into an automated system combining an HPLC apparatus with an in-line radioactivity detector (Flo-one, Radiomatic Instruments, La Queue-Lez-Yvelines, France).

RNA extraction and Northern blot analysis

The DU 145 cell-line was cultured in two 75 cm^2 flasks as described above and plated in RPMI-1640 with 1% DCC. After 48 h, total cellular RNA was isolated by the acid guanidinium thiocyanate–phenol–chloroform method [28], analyzed (20 μg) on denaturing formaldehyde–1% agarose gels, and capillary transferred to nylon Hybond-N filters (Amersham, UK). Filters were baked for 2 h at 80°C, prehybridized

for 6 h at 42°C and hybridized for 24 h at 42°C with 1–1.5 × 10^6 cpm of random primed ^{32}P human types 1 and 2 5α-R cDNA per ml of hybridization solution. Washed filters were exposed for 18 h (type 1) to 4 days (type 2) to X-ray films at −80°C (Hyperfilm-MP, Amersham, UK).

Construction of recombinant baculovirus

The cDNA corresponding to human type 1 5α-R mRNA was isolated from a ph5α45 plasmid vector (kindly provided by Professor D. W. Russell, Dallas, TX) as described previously [13]. The 1.95 kb EcoRI fragment was inserted into the baculovirus transfer vector pEV55[29] at the EcoRI cloning site to yield pEV55-type 1 5α-R plasmid. Transfer of the type 1 5α-R cDNA next to the polyhedrin gene promoter in the AcNPV genome was accomplished following calcium phosphate cotransfection of pEV55-type 1 5α-R and wild-type AcNPV DNA into Sf9 insect cells [30].

Assay of type 1 5α-R activity expressed in the baculovirus-directed insect system

Sf9 cells (0.5–1 × 10^6 cells/ml) were infected with recombinant baculovirus at a multiplicity of infection of 5–10, cultured in 75 cm^2 flasks (Costar) at 27°C, and harvested 4 days post-infection by centrifugation. The pellet was suspended in 50 mM Tris–HCl, 50 mM EDTA (Tris–EDTA buffer), pH 7.4. Cells were then lysed using a pestle B Dounce homogenizer and sonicated for 30 s (Ultrasons Annemasse, France).

An aliquot of 20–50 μl of cell homogenate (ca. 0.15 × 10^6 cells), to which 1 μM T and 0.6 × 10^6 cpm [^{3}H]T were added, was made up to a final volume of 250 μl. The reaction, performed at 37°C, was started by introducing 5 mM NADPH and was stopped after 10 min by immersing the reaction tube in liquid nitrogen. The tube was stored at −20°C until steroid assay. A Lineweaver–Burk plot was obtained by incubation with a range of substrate concentrations (0.1–10 μM T). Inhibition of enzyme activity was determined after incubation with a wide range of inhibitor concentrations: 4-MA (1–500 nM); finasteride (10–3000 nM) and LSESr 1–100 μg/ml. Data were analyzed by the program of Chou and Chou ("Dose-effect analysis with microcomputers", Biosoft, Cambridge, England).

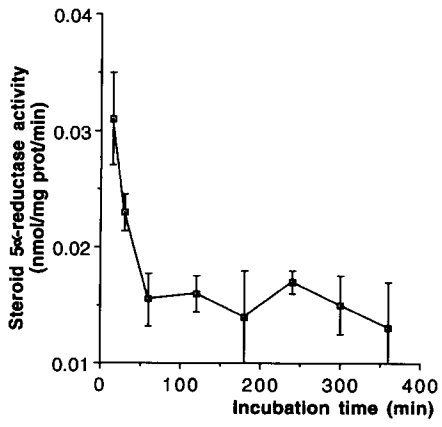

Fig. 1. Time-course of 5α-R activity in DU 145 cells. Subconfluent DU 145 cells were incubated with 0.1 μM T and 0.6 × 10^6 cpm [^{3}H]T. Each point is the mean of at least three experiments.

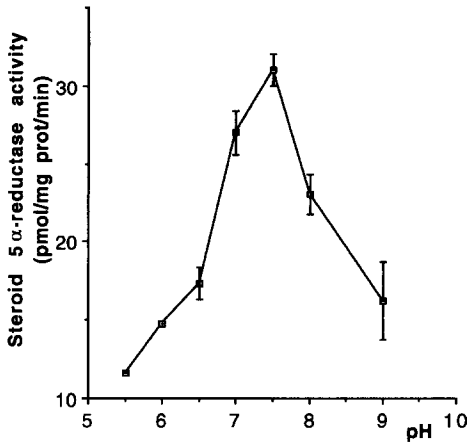

Fig. 2. pH-dependence of 5α-R activity in the DU 145 cell-line. Enzyme activity was assayed as indicated in Materials and Methods. The above plot is typical of the results obtained in two separate experiments.

RESULTS

Characterization of 5α-R activity in DU 145 cells

On incubation of DU 145 cells with [^{3}H]T, 5α-R activity remained stable from 1 h onwards (Fig. 1). A 1 h incubation time was therefore selected in all subsequent experiments. Measurement of 5α-R activity in DU 145 cells as a function of pH (Fig. 2) revealed a narrow enzyme activity peak with a maximum at pH 7.4, thus suggesting that the 'basic' isoform of the enzyme is functional in these cells.

Two types of experiments were performed to confirm the presence of 'basic' type 1 5α-R activity in the DU 145 cell-line. The first experiment yielded indirect evidence and involved the use of specific inhibitors of 5α-R activity as molecular probes. 4-MA and finasteride, when added to the DU 145 cell culture medium, led to a concentration-dependent reduction in 5α-DHT (Fig. 3). A 50% decrease was obtained with 2.8 ± 0.2 nM of 4-MA and 264 ± 55 nM of finasteride. This 90-fold difference in activity is in agreement with published results for inhibition of the 'basic' type 1 enzyme by these two inhibitors [6]. In the second set of experiments, total RNA content from cells cultured for 48 h in serum-free medium was examined in Northern blots. Only the presence of the mRNA for 'basic' type 1 5α-R was detected (Fig. 4).

'Basic' type 1 5α-R expressed in Sf9 insect cells

The activity of 'basic' type 1 5α-R expressed in the baculovirus-directed insect cell system decreased rapidly with time whatever the NADPH concentration (not shown). Conditions of 10-min incubation with 5 mM NADPH were considered to be the most appropriate for subsequent experiments since these curtailed

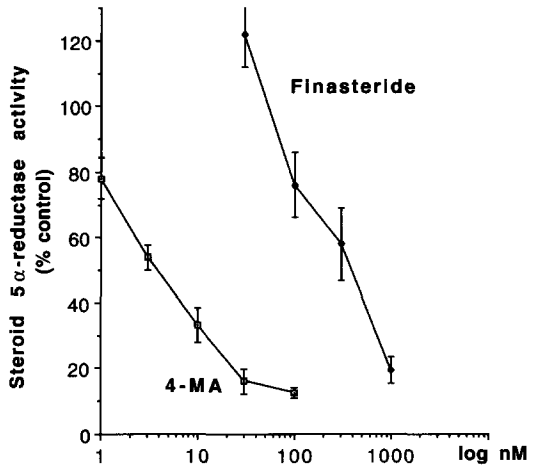

Fig. 3. Inhibition of 5α-R activity in DU 145 cells by 4-MA and finasteride. Cells were incubated with 0.1 μM T, 0.6 × 10^6 cpm [^{3}H]T, and inhibitor. Results are expressed as percent control (without inhibitor). Each point is the mean of 6 experiments.

NADPH degradation and provided a relatively high level of enzyme activity. Activity was recorded at pH 7.5–7.8 but not at pH 5.0.

The type 1 5α-R expressed in the baculovirus-directed insect cell system was inhibited more markedly by 4-MA than by finasteride (IC_{50}s = 9.25 ± 2.58 nM and 400 ± 100 nM, respectively) [Fig. 5(A)], the activity ratio being similar (90-fold) to that recorded above in DU 145 cells. The *n*-hexane extract of *Serenoa repens* fruit (LSESr) also inhibited type 1 5α-R. Its IC_{50} was 4 ± 1 μg/ml but cannot be expressed in nM terms because of the presence in the extract of components (free fatty acids and sterols) of different molecular weight. To circumvent this problem, another team [41] adjusted the inhibition curves comparing finasteride and LSESr in the rat as a function of the therapeutic dose recommended by the manufacturers in the pharmacopeia for the treatment of BPH

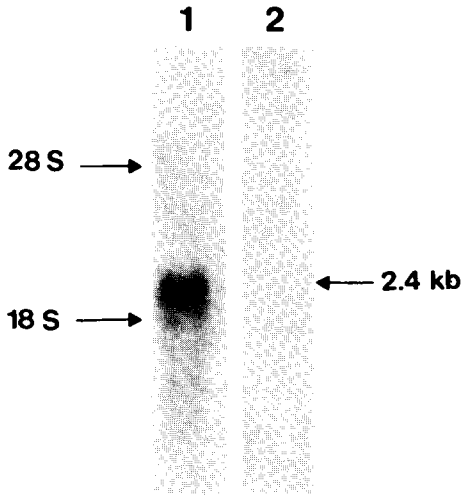

Fig. 4. Northern blot analysis of total RNA from DU 145 cells performed as described under Materials and Methods. The positions of 18 and 28S rRNA are indicated on the left-hand side.

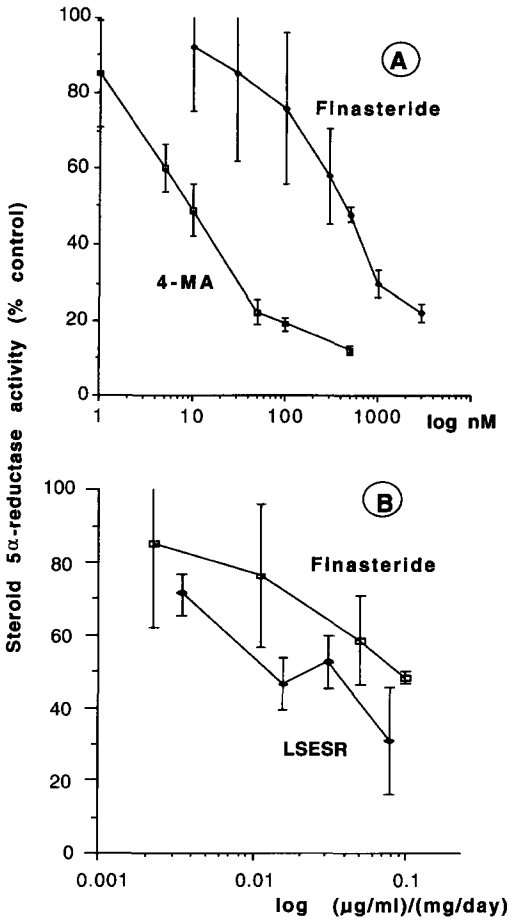

Fig. 5. Inhibition by (A) 4-MA and finasteride and (B) by finasteride and LSESr of 'basic' type 1 5α-R activity expressed in infected Sf9 cells. In B, concentrations have been adjusted to the recommended doses for BPH treatment (5 mg/day finasteride, 320 mg/day LSESr).

(5 mg/day finasteride, 320 mg/day LSESr). On performing a similar adjustment in our studies, LSESr proved to be a 3-fold more potent competitor [Fig. 5(B)]. Lineweaver–Burk plots of enzyme activity vs substrate concentration in the presence or absence of 10 nM 4-MA and 500 nM finasteride established that these 4-azasteroids are competitive inhibitors with K_i values of 8.4 ± 23 and 330 ± 9 nM, respectively (Fig. 6). On the other hand, the LSESr inhibition plot was non-Michaelian and reflects a different mechanism of inhibition of type 1 5α-R.

DISCUSSION

The present study has, first of all, established that DU 145 cells preferentially express only one of the two 5α-R isozymes so far identified and that this is the 'basic' type 1 isoform. The evidence for the presence of this isozyme was 4-fold. (a) Enzyme activity was detected at a basic pH only; (b) blot analysis of RNA revealed the presence of type 1 enzyme mRNA which hybridized with the cDNA of type 1 5α-R only; (c) in tests with 5α-R inhibitors used as probes, a low inhibitory potency was recorded for finasteride compared to

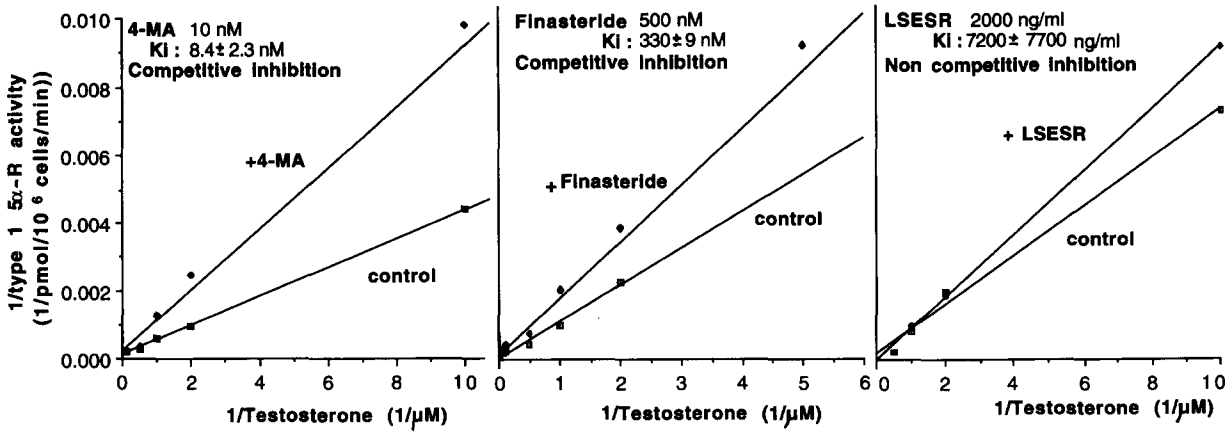

Fig. 6. Double reciprocal plots of the inhibition by 10 nM 4-MA, 500 nM finasteride and 2 μg/ml LSESr (IC_{50}s) of 'basic' type 1 5α-R activity expressed in infected Sf9 cells.

4-MA, which was a 90-fold more potent inhibitor, in line with the potency ratio already recorded for these 4-azasteroids with regard to inhibition of the 'basic' type 1 human isozyme [6]; (d) 4-MA was a more potent inhibitor of the type 1 5α-R expressed in a baculovirus-directed insect cell system than finasteride. If one compares the absolute IC_{50} values of finasteride for the enzyme expressed in our insect cells and in simian COS-M6 cells [6], one notes IC_{50}s of 400 and 900 nM, respectively and K_is of 330 and 230 nM, respectively.

In a previous study performed on BPH homogenates, we recorded an optimum acidic pH for 5α-R activity in line with the work of several teams but not with the practice of 5α-R activity measurement by others (reviewed in [32, 33]). In a recent paper [9], it has been hypothesized that a second 5α-R subunit or protein could explain the absence of an enzyme with a neutral to basic pH optimum in BPH extracts, despite the fact that this tissue contains the mRNA from which the cDNA was derived. To us, this second subunit or protein hypothesis seems most attractive since we have in the past shown that BPH and prostate cancer 5α-R can exert a hysteretic non-Michaelian regulatory behavior [5, 34]. At the time, we offered the following speculative explanation for this behavior: "a slow association/dissociation process in a polymeric enzyme or slow association or dissociation of a modifier on the enzyme" [5] as being the most common mechanisms underlying slow transients and apparent cooperativity in hysteretic enzymes [35] and suggested that it might be of physiological relevance in regulating tissue DHT concentrations in response to wide oscillations in circulating T. Deregulation of 5α-R activities by environmental cellular changes might thus account for high intratissular DHT levels in BPH in spite of apparently unmodified mean 5α-R activity compared to normal tissue. This therefore begs the question whether BPH treatment necessitates an inhibitor or regulator of 5α-R isozymes.

Using the human recombinant 'basic' type 1 5α-R, the present study has established that, of the 4-azasteroids, 4-MA is a more effective competitive inhibitor than finasteride but has also revealed that, at concentrations adjusted to the recommended doses for BPH treatment, LSESr (320 mg/day) is a 3-fold more effective inhibitor than finasteride (5 mg/day). Interestingly, however, inhibition by LSESr is non-competitive indicating that its action is more in line with the notion of enzyme regulation as outlined in our previous kinetic studies [5].

An analysis of the literature strongly suggests that this inhibitory-cum-regulatory action of LSESr might be partly explained by its lipid components. 5α-R is an enzyme located in the nuclear membrane [36, 37] whose solubilization requires lipid [38]. In investigations on brain 5α-R, the androgen metabolite profile in the white matter was considered to be influenced by the amount of lipid [39] and in our studies differences were found in the metabolite recorded after enzyme inhibition by LSESr and 4-azasteroids (unpublished data). Phospholipids (phosphatidyl-serine, -choline, -ethanolamine and -inositol) with acyl chains of different lengths (C8 to C18) exert a range of stimulatory/inhibitory activities on 5α-R in testicular microsomes [40]. Finally, certain specific aliphatic unsaturated fatty acids, such as, for instance, γ- and α-linolenic, arachidonic, palmitoleic and oleic acids, as well as extracts besides LSESr from other palmettos are inhibitors of 5α-R [41]. The potency of the latter varies substantially with the method of extraction [42].

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