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Kinetic Analysis of Steroid 5α-Reductase Activity at Neutral pH in Benign Prostatic Hyperplastic Tissue: Evidence for Type I Isozyme Activity in the Human Prostate

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In human benign prostatic hyperplastic (BPH) tissue homogenates 5α-reduction of testosterone was examined at neutral pH. As Lineweaver-Burk and Eadie-Scatchard plots of estimated initial velocities against a wide range of substrate concentrations of 2 nM to 3.2 μM were non-linear, the existence of two 5α-reductase isozymes in this tissue was surmised. Indeed, enzyme parameters at pH 7.0 suggested the presence of two isozymes with affinity constants of 1995 and 11.8 nM, characteristic of the well established human steroid 5α-reductase isozymes type I and II, respectively. The physiological roles of these isozyme activities remain puzzling. The specific activities, Vmax, of these subtypes indicated an approx. 6-fold higher maximum velocity of type I than of type II 5α-reductase in the human hyperplastic prostate at this pH. In contrast, the efficiency ratios, Vmax/Km, demonstrated that the type II isozyme had a nearly 27 times higher potential in vivo activity than the type I isozyme, and is therefore most probably quantitatively responsible for dihydrotestosterone formation at physiological testosterone levels in this tissue at neutral pH. This is the first full paper on type I 5α-reductase activity in human BPH tissue. Copyright © 1996 Elsevier Science Ltd.

INTRODUCTION
Steroid 5α-reductase (E.C. 1.3.99.5) is a NADPH-dependent enzyme capable of 5α-reducing a number of steroids with a 4,5 double bond and a 3-oxo group, including glucocorticoids, progestogens, mineralcorticooids, androgens [1] and non-androgens such as the pheromone precursor 4,16-androstadien-3-one [2, 3]. The 5α-reduction product of testosterone (T), dihydrotestosterone (DHT), has been implicated in the pathogenesis of benign prostatic hyperplasia (BPH) [4].

Earlier studies on the 5α-reductase enzyme characteristics in prostatic epithelium and stroma revealed a difference in affinity constants, which might indicate the existence of two isozymes in the human prostate [5-8]. As it is now well established that there are two human 5α-reductase isozymes, i.e. type I and type II, with distinct pH-optima, tissue distribution, affinity constants and sensitivity to inhibitors [as reviewed in 1], one might assume that the human prostate indeed contains both of these isozymes.

Prostatic 5α-reductase type II mRNA, -protein and -activity have been detected using cDNA probes [9, 10], immunoblotting and immunocytochemistry [10-12], and pH profiles of 5α-reductase activity [1, 13], respectively. In patients with 5α-reductase type II deficiency (male pseudohermaphroditism) prostate development is impaired, indicating the importance of this 5α-reductase isozyme for the growth and development of the human prostate [14, 15]. It is therefore intelligible that a specific type II isozyme inhibitor, finasteride, has been developed for treatment of benign prostatic hyperplasia.

Data concerning the presence of the type I 5α-reductase in human prostate, however, are conflicting; the first human 5α-reductase cDNA was derived from prostatic tissue [13, 16] and was shown to code for the type I isozyme. Using cDNA probes, mRNA for the type I 5α-reductase has been found in the human prostate [9], whereas other investigators failed to
demonstrate type I 5α-reductase mRNA in this tissue [10]. Furthermore, immunoblotting studies using specific antibodies against the 5α-reductase protein denies type I immunoreactivity [10, 12]. On the other hand, apparent affinity constants for 5α-reductase found in human prostatic tissues are consistent with type I activity [17] and preliminary data have been published in abstract form indicating both type I and type II 5α-reductase activity in this tissue [18].

There is growing evidence that both 5α-reductase isozymes operate at neutral pH [19]. By incubating homogenates of rat prostate and epididymis with a wide range of substrate concentrations, we were recently able to measure both rat isozymes simultaneously [20]. In the present study we applied the same procedure to human BPH homogenates. A careful analysis of 5α-reductase activity at pH 7.0 showed non-linear Lineweaver-Burk and Eadie—Scatchard [21] plots of initial velocity against substrate concentration. This could be adequately explained by the presence of two isozymes in BPH tissue. The kinetic characteristics of these isozymes were in concordance with those of the well established human steroid 5α-reductase type I and II isozymes. Both the type I and type II isozyme specific activities and potential in vivo activities were determined.

MATERIALS AND METHODS

Materials

$[\text{1,2,6,7-}^{3}\text{H}]\text{T} \text{ (3.74 TBq/mmol)}$ and $[\text{1α,2α(n)-}\text{3H}]\text{-17β-hydroxy-5α-androstan-3-one (DHT)} \text{ (2.00 TBq/mmol)}$ were purchased from Amersham (Amersham, U.K.). $[\text{9,11-3H}]\text{5α-androstan-3α,17β-diol (1.48 TBq/mmol)}$ was obtained from Du Pont-New England Nuclear (Boston, MA). All radiolabelled steroids were purified by high performance liquid chromatography (HPLC, see below) before use. Non-labelled T was purchased from Steraloids (Wilton, NH). Diethylether (p.a.), $n$-hexane (LiChrosolv) and 2-propanol (LiChrosolv) were purchased from Merck (Darmstadt, FRG). All other chemicals used were of analytical grade.

Protein levels were determined by a modification of the method of Lowry et al. [22] against a standard bovine serum albumin (OHRD 20/21, Hoechst-Behring, Marburg, Germany). The assay was modified for microtiter-plates and had a sensitivity of 25 μg per well.

Buffers

Homogenization buffer consisted of 20 mM phosphate (Merck), 1 mM monothioglycerol and 0.25 M sucrose (Merck), pH 7.0. The incubation buffer consisted of 200 mM Tris (2-amino-2-hydroxymethylpropane-1,3-diol, Merck) and citric acid monohydrate (Merck), pH 4.5–8.0 or pH 7.0, and 2 mM NADPH tetrasodium salt (Merck).

Tissue preparation

Human benign prostatic hyperplastic tissues were obtained from 15 different patients, age 53–69 y, undergoing transurethral resection. Prostatic tissue chips were placed in liquid nitrogen for transport to the laboratory. Aliquots of each specimen were sent to the Department of Pathology for histological confirmation of BPH. Tissues were kept at −80°C or processed immediately. All subsequent procedures were performed on ice. Tissues (9.3 g total w/w) were pooled, thawed, and minced with razor blades into small pieces. Minced tissue was homogenized in ice-cold homogenization buffer with a 7 ml Dounce tissue grinder (Kontes Glass Co., Vineland, NJ) with a loose and a tight fitting pestle. The homogenate was filtered twice through nylon netting of 50 and 140 mesh respectively to remove cell debris. By this procedure—without centrifugation—a full homogenate with nuclei and cytosol is obtained. The pooled BPH homogenate was diluted with homogenization buffer to 5.4 mg protein/ml.

5α-reductase assay

Radiolabelled T in ethanol was brought to final concentration (2 nM–3.2 μM) by isotopic dilution with non-labelled steroid in Pyrex culture tubes (borosilicate glass, 12 x 75 mm, Corning Inc., Corning, NY). Ethanol was evaporated under a mild nitrogen stream at room temperature. Incubation buffer (800 μl) was added and the tubes were put into a shaking water bath at 37°C at least 10 min before the start of the incubation to ensure the substrate was dissolved (97%). A tube with the homogenate (50–100 μl) and the appropriate amount of cofactor (NADPH, 2 mM final concentration) was diluted to 200 μl with 100–150 μl incubation buffer and kept on ice. The incubation was started by adding 200 μl of the homogenate and cofactor mixture to the pre-heated tubes with substrate. After 10–30 min the incubation was terminated by adding 100 μl of 3 M NaOH. This 5α-reductase assay protocol was optimized for rat tissues (prostate and epididymis) in an earlier paper [23]. The assay was evaluated for BPH tissues, and was checked for linearity in time and protein concentration. T metabolism was not allowed to exceed 15% by varying enzyme concentration and incubation time. To extract metabolites, 4 ml of ice-cold diethylether was added, and the tubes were capped and shaken. The water phase was frozen in an alcohol bath with dry-ice, the organic phase decanted and evaporated under nitrogen. Metabolites were dissolved in 100 μl hexane for HPLC.

HPLC

Metabolized steroids were separated on a Hibar LiChrosorb Diol-column (length 250 mm, 5 μm, Merck), equipped with a guard column (Resolve Silica, Waters Corp., Milford, MA). The HPLC-system
included a Waters 610 Fluid Unit, a Waters 600E System Controller and a Waters U6K injector. The isocratic flow of the mobile phase (hexane–propanol 96:4, v/v) was 1.5 ml/min at a pressure of 680 psi. Radioactivity was monitored with a FloOne Beta Radiomatic A500 radio-chromatography detector (Packard-Canberra Benelux, Tilburg, The Netherlands) with a 500 µl cell and a liquid scintillation flow of 1.5 ml/min (Aqua-Luma, Lumac-LSC, Olen, Belgium). The counting efficiency for tritium was 47%.

Calculation of enzyme characteristics

Velocities were plotted against T concentration, and $K_m$ and $V_{max}$ were calculated using a non-linear regression procedure based on a Michaelis-Menten equation for two isozyme activities. A double reciprocal plot of the obtained estimated initial velocities against substrate concentration was used. This Lineweaver-Burk plot appeared to be non-linear at high T concentrations. Furthermore, an Eadie-Scatchard plot of velocity over substrate concentration ($V/S$) against velocity ($V$) was used for a more even weighting of points [Fig. 2(A)]. This plot was clearly non-linear and indicated the presence of isozyme activities. Two 5α-reductase activities with different $V_{max}$ and $K_m$ could be calculated using the same non-linear regression procedure with fitting to least squares. For the use of the $V_{max}/K_m$ ratio as an index of potential enzyme activity one has to consider that endogenous T concentrations are much lower than the $K_m$ of either 5α-reductase subtype [5]. Therefore applying $K_m > [S]$ in the Michaelis-Menten equation gives:

$$v \approx \frac{V_{max}}{K_m} [S].$$

So, at physiological T concentrations, the enzyme reaction velocity is proportional to $V_{max}/K_m$.

RESULTS

Validity of the 5α-reductase assay

In this study we investigated the 5α-reductase characteristics in human benign prostatic hyperplastic tissue homogenates. The 5α-reductase assay we applied was optimized and its validity checked in rat tissues as reported in an earlier paper [23]. The validity of this protocol was confirmed for the present study in BPH homogenates. In the absence of cofactor, the enzyme was unstable at 37°C. This deterioration could be countered by adding cofactor during the preincubation. With 2 mM NADPH, the enzyme activity was stable for at least 30 min at 37°C. This stabilization of enzyme activity by preincubating with cofactor has been reported earlier by others [24, 25]. Furthermore, the

initial burst in the time course of the 5α-reduction of T, reported by others in BPH particulate fractions [26] and by us in rat epididymis and prostate [23], was also countered with the applied protocol in the present study. This procedure led to a linear time-course during the incubation period for at least 30 min, allowing for the estimation of initial velocities. Finally, 10–100 µl of the diluted homogenate (0.054–0.54 mg protein) was incubated under the established conditions mentioned in the Materials and Methods section. The velocities were linear for the entire protein concentration range tested (results not shown).

pH optimum

A pH profile of enzyme activity was made over the pH range 4.5–8.0 using 4 substrate concentrations (Fig. 1). For purpose of comparison, the initial velocity at the optimum pH is arbitrarily set at 100% for each of the T concentrations. At T concentrations of 100, 200 and 1000 nM, the optimum of initial velocity was found at pH 5.5. However, at a lower T concentration of 10 nM, the optimum was at pH 6.0.

Isozyme activities at neutral pH

At pH 7.0 the initial velocities were estimated over a wide concentration range of 2 nM–3.2 µM T [Fig. 2(A)]. As the double reciprocal plot of these data was non-linear at high T concentrations [Fig. 2(B)], a non-linear fit to least squares was used and the enzyme parameters of two 5α-reductase isozymes could be calculated. An Eadie-Scatchard plot of $V/S$ against $V$ was used for a more even weighting of points [Fig. 2(C)] and was more clearly non-linear. One subtype was calculated with a $V_{max}$ of 50.8 fmol/(min-mg protein) and a $K_m$ of 11.8 nM, which is characteristic of the established type II isozyme at this pH. The other subtype could be defined as having a $V_{max}$ of

% of maximum activity

Fig. 1. pH-Profiles of 5α-reductase activity at 2 mM NADPH and with (•) 10 nM, (△) 100 nM, (■) 200 nM and (○) 1000 nM T as substrate. The optimum pH for 5α-reductase activity in human BPH tissue depends on the substrate concentration chosen. For purposes of comparison at each concentration the optimum activity was arbitrarily set at 100%.
313.1 fmol/(min·mg protein) and a $K_m$ of 1995 nM, corresponding to $K_m$ values reported for the type I isozyme. The efficiency ratio $V_{\text{max}}/K_m$ of the alleged type I isozyme was $0.16 \cdot 10^{-6}$ l/(min·mg protein), whereas the type II isozyme had an approx. 27-fold higher $V_{\text{max}}/K_m$ ratio of $4.30 \cdot 10^{-6}$ l/(min·mg protein). For purpose of comparison, the $V_{\text{max}}$ and the $V_{\text{max}}/K_m$ ratios of both isozymes at pH 7.0 are shown in Fig. 3. Although the $V_{\text{max}}$ of the type I 5α-reductase isozyme was 6.2-fold higher than that of the type II, it yet appeared that the potential in vivo activity at pH 7.0 is mainly attributable to the type II isozyme, as evidenced by its much higher $V_{\text{max}}/K_m$ ratio.

DISCUSSION

In this study we investigated the 5α-reductase characteristics of human benign prostatic hyperplastic tissue homogenates. In a previous paper [23] we described deterioration of enzyme activity at both pH 5.0 and at pH 7.0 in the absence of cofactor at 37°C in rat prostate and rat epididymis. The enzyme activity was stable for 30 min at 37°C with 2 nM NADPH. This also applied to 5α-reductase activities in BPH homogenates described in the present paper. The initial burst in the time-course of 5α-reductase activity [23, 26] was not present with the protocol applied in this study. Furthermore, the activity was linear over the whole protein concentration range tested. Therefore, we conclude that the values we established here are reasonable estimates of initial velocity.

We established the pH-optimum for 5α-reductase activity in BPH homogenates. This pH-optimum of 5α-reductase activity in human prostate has been reported to vary from as low as pH 5.0 [13], to pH 5.5 [26] or even 7.0 [8, 17]. In this study we measured the pH dependency of 5α-reductase activity at substrate concentrations ranging from 10 to 1000 nM T. At the
higher T concentrations (100–1000 nM), the pH optimum was found at 5.5. At the lowest T concentration tested (10 nM), however, the optimum was at pH 6.0. Thus, the pH-optimum strongly depends on the substrate concentration chosen. As the affinity constant for 5α-reductase activity depends substantially on pH [19, 27], the pH-optimum found will greatly influence reported K_m values for 5α-reductase in BPH tissue. This might—in part—explain the wide range of physiological pH for both isozymes [19].

The existence of type II 5α-reductase in human prostatic tissue is now well accepted [9–13]. In patients with a type II 5α-reductase deficiency (male pseudohermaphroditism) the prostate is atrophic [14, 15]. In these patients the type I isozyme is unaffected, indicating the crucial function of the type II isozyme in the development of the human prostate. Data concerning type I 5α-reductase diverge, as some authors deny the existence of the type I isozyme in the human prostate [10, 12], whereas others claim its presence [9, 13]. Preliminary data has been published reporting equal amounts of type I and type II activity in human prostatic tissue [18]. We describe here the presence of two isozymes in a full homogenate of BPH tissue, by applying high T concentrations and by using an Eadie–Scatchard plot of the obtained data. This plot is particularly suitable for identifying two isozymes as it leads to a more even weighting of points as compared to the Lineweaver–Burk plot [21]. The affinity constants for these isozymes are typical for the type I and II 5α-reductase subtypes at neutral pH as reported in literature [16, 19, 27]. When V_max is taken as a measure of enzyme concentration, our results imply that there is an approx. 6-fold higher amount of type I 5α-reductase than of type II at pH 7.0 in the human hyperplastic prostate. However, one has to be cautious regarding these results; to preserve all cellular components we did not centrifuge our homogenate, but filtered the preparation through nylon netting which might have led to a preferential recovery of epithelial components [7]. Stroma and epithelium do probably not exhibit the same 5α-reductase isozyme composition [5–8]. Thus a better recovery for the epithelial components in this study would have led to a difference in relative isozyme concentrations found. Furthermore, unlike this paper, most studies use a centrifugation step in their experimental procedure to remove cell debris. However, whole nuclei will also be lost at 800 g, and as there might be a difference in intracellular localization between both 5α-reductase subtypes, more of a nuclear bound isozyme could have been detected with our protocol than in these other studies.

For enzyme quantification several definitions can be used: the maximum velocity at the optimum pH of the isozyme, the V_max at neutral pH—as the acidic pH optimum of the type II 5α-reductase is probably not physiological—or the efficiency ratio V_max/K_m as a measure of isozyme activity, instead of isozyme concentration. All these definitions can, and have been, used. At pH 5.5 a maximum velocity of 0.7 pmol/(min·mg protein) was found, at pH 7.0 the V_max was 0.13 pmol/(min·mg protein), indicating a 5.4-fold higher type II than type I isozyme specific activity (results not shown). Considering our data of isozyme activity at pH 7.0, the V_max/K_m ratios for both isozymes point to a 26.9-fold higher potential in vivo activity for the type II than the type I isozyme in human prostatic tissue at pH 7.0. When this efficiency ratio is used as a measure of isozyme activity, the results suggest that a specific type II isozyme inhibitor, like finasteride, should suffice in decreasing prostatic DHT tissue levels in the clinical management of BPH. However, it has not been unequivocally ascertained whether the premise of Km >> [S] for the use of the efficiency ratio is valid in the microenvironment of the type I isozyme; human prostatic 5α-reductase is reported to be located in the nucleus [28]. One can speculate that the type I isozyme could be active when T is accumulated in the nucleus to a higher substrate concentration, e.g. by binding to the androgen receptor. If this would be the case, the substantial type I 5α-reductase activity reported here might call for an evaluation of a combination therapy with type I and II isozyme inhibitors for BPH [15].

In conclusion, we present here evidence for the presence of a substantial amount of type I 5α-reductase in human benign prostatic hyperplastic tissue homogenates in addition to the well established type II. Nevertheless, although type I has an approx. 6-fold higher specific activity than type II 5α-reductase at neutral pH, it is most probably the type II isozyme that is quantitatively responsible for the formation of DHT in the human prostate, considering the efficiency ratio V_max/K_m of the isozymes in this tissue. However, the intracellular and cell-type specific localization of both isozymes has to be considered so as to shed more light on the possible physiological roles of both isozymes in this tissue. The role of the reported prostatic type I 5α-reductase isozyme in BPH remains intriguing.

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REFERENCES