STUDIES ON THE KINETICS OF THE INTERACTION OF 7α-HYDROXYTESTOSTERONE WITH THE STEROID 5α-REDUCTASE

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ABSTRACT

Microsomal preparations from adult male rat testicular interstitial cells were incubated with tritiated testosterone. Added 7α-hydroxytestosterone, (7α,17β-dihydroxy-4-androsten-3-one), at levels which appear to exist in the adult testis, inhibited production of labelled 5α-reduced steroids in a graded fashion. This interaction is not competitive and occurs only at high substrate levels, such as those found in steroid-producing organs. Relationships to pubertal changes in steroid metabolism are discussed.

INTRODUCTION

The testes of mature rats will produce large amounts of the polar steroid 7α-hydroxytestosterone from testosterone *in vitro* (1,2). Lacroix *et al* (2) found the content of 7α-hydroxytestosterone in the adult rat testis to be about the same as that of testosterone, but when testis fragments were incubated (without LH or HCG), more than twice as much 7α-hydroxytestosterone as testosterone was produced. Chronic HCG treatment induces increased production of 5α-reduced steroids and strong depression of 7α-hydroxytestosterone formation from both endogenous and added radiolabelled precursors in adult rat testis (3). The 7α-hydroxylase is located on the smooth endoplasmic reticulum (4,5). Tested in bioassays, 7α-hydroxytestosterone displays neither androgenic nor anabolic properties (5,6). 7α-Hydroxytestosterone will, however, inhibit testicular steroid metabolizing enzymes like Δ^5-3β-hydroxy-steroid dehydrogenase (7), 5α-reductase

(8) and 3α-hydroxy-steroid dehydrogenase (8) in vitro.

We now report studies on the kinetics and dose-response relationships with the interaction of 7α-hydroxytestosterone and the Δ^4-steroid 5α-reductase.

MATERIALS AND METHODS

Male Sprague-Dawley strain rats (Charles River Breeding Laboratories, Wilmington, MA) weighing 400-430 g were killed by rapid decapitation. The testes were immediately decapsulated and placed in medium 199 (Gibco, Grand Island, N.Y.). Next, the testes were treated with collagenase (Worthington No. 4194, 1 mg/mL, Worthington Corp., Freehold, N.J.), in medium 199 at 37°C with agitation. Bovine serum albumin (Gibco) was present also at a concentration of 1 mg/mL).

After interstitial and tubular elements were separated, tubules and resistant portions of the testis were removed by passing the suspension through three layers of cheesecloth followed by thorough washing. Interstitial cells were collected by centrifugation and washed three times with medium.

Enzyme activity was determined by the method of Sunde, Tveter and Eik-Nes (9). Interstitial cell preparations were suspended in ice-cold buffer (0.05 M Tris, 0.05 M disodium EDTA, 5 mM $MgCl_2$, 0.05 M NaCl, and 0.5 mM mercaptoethanol adjusted to pH 7.4 with NaOH/HCl). The tissue was then homogenized in a glass homogenizer and centrifuged at 800 x g for 20 min at 4°C. The supernatant contained the microsomes and was used in incubation studies. Protein content was measured by the method of Lowry et al (10).

Tritiated testosterone (Amersham, Downers Grove, Ill., labelled in the 1α and 2α positions, 60 Ci/mmol, 50 ng/tube, unless otherwise specified), was dried in conical polypropylene centrifuge tubes with or without 7α-hydroxytestosterone (Medical Research Council of Great Britain or E. Merck, Darmstadt). Buffer (110 µL) was added to each tube together with an NADPH generating system [10 µL $NADP^+$ (2.5 mM), 20 µL glucose-6-phosphate (12.5 mM), and 10 µL glucose-6-phosphate dehydrogenase, type XV (250 µg/mL), all from Sigma Chemical Co., St. Louis, MO]. After addition of buffer and cofactors, the tubes were vortexed and preincubated for 5 min at 37°C in a water bath with shaking. Next, 100 µL testicular supernatant was added to each tube. Incubations were then carried out at 37°C in an atmosphere of 95% O_2-5% CO_2 for 90 minutes unless specified otherwise. Blank tubes were immediately extracted while the experimentals incubated. Next, the reaction was stopped by addition of 7 mL dichloromethane containing nonradioactive carrier steroids. After shaking, the aqueous phase was removed and the organic phase was dried and dissolved in ethanol.

Next, the extracts were spotted on propylene glycol-impregnated TLC plates (11) which were run twice in CCl_4-cyclohexane (9:1 v/v). Zones corresponding to 5α-dihydrotestosterone, 5α-androstane-3α, 17β-diol, 5α-androstane-3β, 17β-diol, androsterone, and 5α-androstane-3,17-dione were removed from the plate, eluted, and counted by liquid

scintillation as a measure of 5α-reductase activity.

Figure 1 presents data in which 200 ng testosterone was present per mL, with variations in 7α-hydroxytestosterone. Means ± S.E.M. are presented; in parentheses are number of determinations per point. The multiple range test of Duncan (12) was used to determine probability.

Figure 2 presents data in which control testosterone varied from 8 to 800 ng/mL. Experimental tubes had, in addition, 800 ng/mL (2.628 μM) 7α-hydroxytestosterone. Data are presented as Lineweaver-Burk plots (13) with number of determinations per line. The lines were fitted by least squares regression with analysis of variance to test significance (14).

RESULTS

Figure 1 shows the effects of adding varying amounts of 7α-hydroxytestosterone to the incubation system. Addition of 400 ng/mL 7α-hydroxytestosterone reduced the conversion of tritiated testosterone to 5α-reduced products to 59.9% of control ($P<0.05$). Addition of 2,000 ng/mL reduced the level to 40.1% ($P<0.01$), and addition of 10,000 ng/mL reduced the level to 24.6% of control ($P<0.05$ vs the 400 ng/mL level). Thus, there is a graded inhibitory effect.

It is recognized that the control microsomal preparation contained an unknown amount of 7α-hydroxytestosterone and that perhaps 20% of the added testosterone was converted to 7α-hydroxytestosterone during the incubation. Thus, the sensitivity to 7α-hydroxytestosterone is probably being underestimated.

Figure 2 shows Lineweaver-Burk (double reciprocal) plots of control and experimental data. The y (vertical) axis is the reciprocal of the velocity as pg/μg/h; the x axis is the reciprocal of the micromolar concentration of substrate. The control (lower line) has a regression equation of: $y=0.1158+7.1725\times10^{-2}\ x$ (based on 21 determinations ranging from 8 to 800 ng/mL testosterone). The experimental line (with 800 ng/mL 7α-hydroxytestosterone) is based on 23 determi-

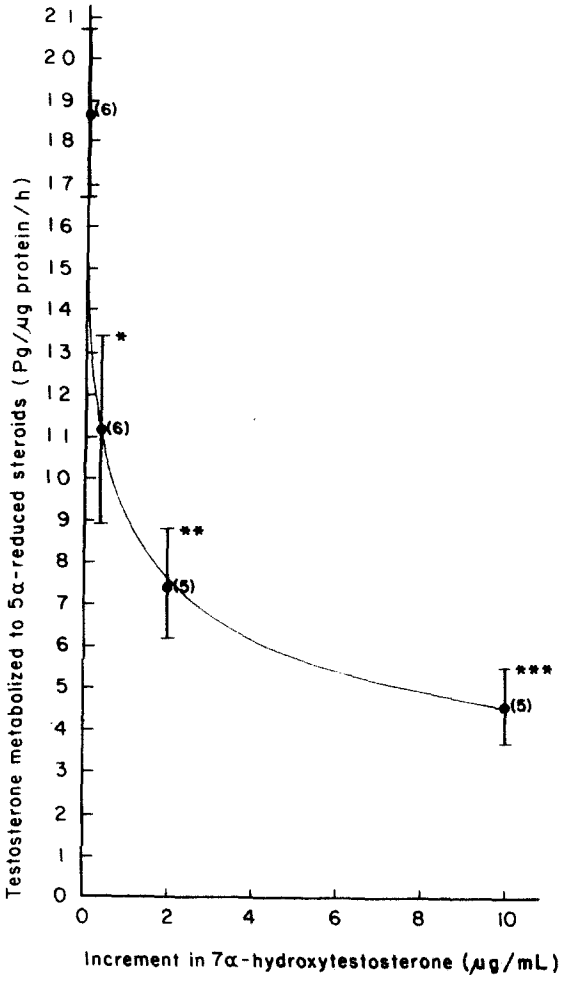

Figure 1. Effect of increasing amounts of 7α-hydroxytestosterone on production of 5α-reduced androgens. * Significantly different from control, $P<0.05$. ** Significantly different from control, $P<0.01$. *** Significantly different from 0.4 ng/mL level, $P<0.05$.

nations and has a regression line of $y=0.5519+7.1617\times10^{-2}x$. The Michaelis constants (Km) are 0.619 μM (control) and 0.130 μM (experimental). These lines are significantly different ($P<0.05$), but both are significantly related to testosterone concentration ($P<0.001$). This pattern of two parallel lines is clearly not compatible with

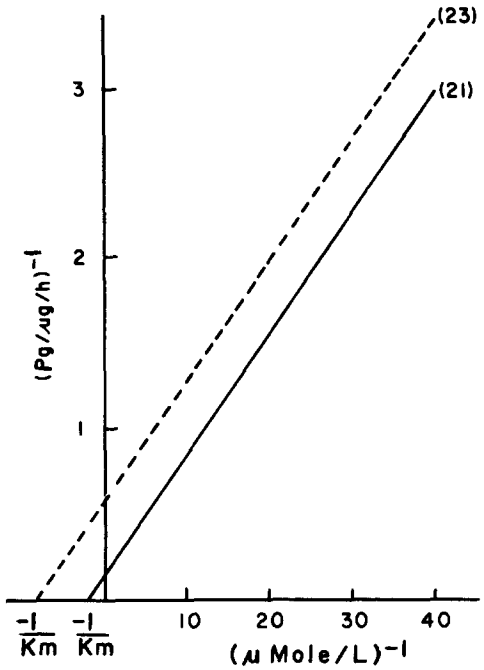

Figure 2. Double reciprocal (Lineweaver-Burk) plots. The solid line represents testosterone alone. The broken line shows the effect of 800 ng/mL added 7α-hydroxytestosterone on 5α-reductase activity.

competitive inhibition.

Linearity of 5α-reductase activity with time in this system was up to 180 min when 400 ng/mL testosterone was used (data not presented). The optimal time for demonstrating an effect of 7α-hydroxytestosterone (400 ng/mL) appears to be around 90 min (19.0% of control, $P<0.002$).

Enzyme activity was positively related to protein content and linear up to the levels used in the previous figures (300-400 μg/assay tube), but declined to insignificant levels with increased microsomal content. 7 α-hydroxytestosterone is without androgenic or anabolic

properties (5,6) and appears to be metabolized slightly or not at all by 5α-reductase (Mittler, unpublished); therefore, substrate or cofactor depletion did not influence these results.

DISCUSSION

Previous reports (15) showed that half or more of the 7α-hydroxytestosterone produced in the testis originates from the interstitial fraction (almost entirely Leydig cells) and that total levels (1,2) are high enough to postulate that 200-400 ng of 7α-hydroxytestosterone/mL are reasonable values for Leydig cell cytoplasm. Since fluid flows to the lumen of the seminiferous tubules, and since some 7α-hydroxytestosterone is produced in the tubules (15) it seems reasonable to postulate that the higher levels of that steroid used in this study may also be found in at least some cells in the tubules.

Similarly, Turner et al (16) reported that testis interstitial fluid contained a mean of 73.1 ng testosterone and 4.9 ng 5α-dihydrotestosterone per mL. Seminiferous tubule fluid content was 50.2 ng testosterone and 1.2 ng 5α-dihydrotestosterone per mL. Therefore, the values for the inhibitory interaction reported here may plausibly be considered to act in vivo.

In contrast, when 800 ng 7α-hydroxytestosterone was matched by 8 ng/mL testosterone (a level that might be found in the prostate) there was no significant inhibition. Thus, this "uncompetitive" inhibition may operate only in the testis (or conceivably adrenal or ovary).

The "uncompetitive" inhibition which appears from the Lineweaver-Burk plots can reflect destruction of enzyme activity, but when the preparation of microsomes was incubated with 7α-hydroxytestosterone before addition of tritiated testosterone, there was no evidence of

inactivation with respect to the appropriate controls (data not presented). Interaction with an allosteric mechanism is a plausible model for these results.

These experiments may underestimate the inhibitory effect of 7α-hydroxytestosterone since the microsomal preparations contain at least some, and make more, during incubation, i.e. the controls were really not without 7α-hydroxytestosterone. Also, androgens stimulate growth and differentiation in the seminiferous tubule. Conversion of testosterone to 5α-dihydrotestosterone markedly enhances its biological activity in the prostate (17) and presumably in any organ containing both 5α-reductase and androgen receptors. Therefore, 5α-dihydrotestosterone may reasonably be assumed to stimulate the production of more 5α-reductase activity and more 5α-dihydrotestosterone. This amplification would be reversed by any inhibitor of 5α-reductase. Such a "positive feedback" has been described in pubic skin fibroblasts (18) and cultured ovarian granulosa cells (19).

We conclude that the amounts of 7α-hydroxytestosterone reported by others (1,2,4,5) to be present in the adult testis (but not immature testis) are sufficient to inhibit the 5α-reductase active on testosterone and to account for the reduction (20) in the ratio of 5α-dihydrotestosterone and 5α-androstane-3α, 17β-diol to testosterone which occurs with puberty in the male rat. However, we cannot exclude participation of other mechanisms.

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