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C-$_{19}$-STEROID 7α-HYDROXYLATION BY RAT TESTES. ISOLATION AND IDENTIFICATION OF: 7α,17β-DIHYDROXY-5α-ANDROSTAN-3-ONE, 5α-ANDROSTAN-3α,7α,17β-TRIOL AND 5α-ANDROSTANE-3β,7α,17β-TRIOL

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SUMMARY

From incubations of testosterone with rat testicular homogenates in the presence of a NADPH-generating system, the following 7α-hydroxylated metabolites could be isolated and identified: 7α,17β-dihydroxy-4-androsten-3-one (7α-hydroxy-testosterone), 7α-17β-dihydroxy-5α-androstan-3-one (7α-hydroxy-Dht), 5α-androstan-3α,7α,17β-triol (7α-hydroxy-3α-A'DIOL) and 5α-androstane-3β,7α,17β-triol (7α-hydroxy-3β-A'DIOL). To our knowledge this is the first demonstration of the formation of 5α-reduced-7α-hydroxylated metabolites of testosterone in the male gonad. These 5α-reduced-7α-hydroxylated metabolites could also be isolated after incubations of 5α-androstane-3α,17β-diol (3α-A'DIOL) with testicular homogenates in the presence of a NADPH-generating system.

Measured as the sum of 7α-hydroxy-testosterone, 7α-hydroxy-Dht, 7α-hydroxy-3α-A'DIOL and 7α-hydroxy-3β-A'DIOL formed using testosterone as substrate, total 7α-hydroxylase activity was six times higher in testes of mature rats than in testes from animals 23 days old. With 3α-A'DIOL as substrate total 7α-hydroxylase in the mature testis was about three times greater than in the sexually immature testis.

INTRODUCTION

The testis of mature rats will produce large amounts of 7α-hydroxy-testosterone from testosterone *in vitro* [1–3]. Tested in bioassays 7α-hydroxy-testosterone displays neither androgenic nor anabolic properties [4, 5], but it will inhibit testicular metabolizing enzymes like Δ5-3β-hydroxy steroid dehydrogenase [6], 5α-reductase and 3α-hydroxysteroid oxidoreductase [3] *in vitro*.

Testicular production rate of 7-hydroxy-testosterone in the adult rat can equal that of testosterone, but the testis of the sexually immature rat produces only minute amounts of 7α-hydroxy-testosterone [2, 3]. Rat testicular 5α-reductase decreases during puberty [2, 3] and 5α-reduced metabolites of testosterone predominate quantitatively in the immature rat testis. 7α-Hydroxylated metabolites of 5α-reduced androgens are produced by rat and human prostate [7, 8], canine perianal gland [9], rat pituitary gland [10] and rat liver [11]. To our knowledge no information is available concerning testicular 7α-hydroxylation of 5α-reduced testosterone metabolites. The purpose of this study was to investigate whether such metabolites are formed by the rat testis, and to determine whether measurements of this class of steroids could determine a new pattern in testicular 7α-hydroxylation activity between sexually immature and mature rats [2, 3].

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MATERIALS AND METHODS

Animals

Wistar rats of different ages were used. The animals were purchased from Institutt for Folkehelse, Oslo, and kept in the animal quarters at Regionsykehuset in Trondheim for a minimum of 3 days before experiments were started. The animals were exposed to controlled light (14 h light and 10 h darkness) and temperature (19–21°C) conditions. Rat chow and tap water were provided *ad libitum*.

Materials

[1α,2α(*n*)-^{3}H]-Testosterone (SA 53 Ci/mmol), [1α,2α(*n*)-^{3}H]-17β-hydroxy-5α-androstan-3-one (SA 60 Ci/mmol and [1α,2α(*n*)-^{3}H]-5α-androstan-3α,17β-diol (SA 41 Ci/mmol) were purchased from the Radiochemical Centre, Amersham, England. Scintillation fluid (Scint Hei-I) was obtained from F. Heidenreich, Oslo, Norway, and was diluted with absolute ethanol (2/100 v/v). Unlabelled steroids were delivered by Steraloids Inc., U.S.A., except for 7α-hydroxy-testosterone which was kindly supplied by Dr K. Irmscher, E. Merck, Darmstadt, Germany. 7α-Hydroxy-Dht, 7α-hydroxy-3α-A'DIOL and 7α-hydroxy-3β-A'DIOL were synthetized from 7α-hydroxytestosterone. Synthesis, and characterization of these steroids will be published elsewhere [12].

Labelled steroids were purified by thin layer chromatography (TLC). TLC was performed on 20 × 20 cm silica gel-60 F254 plates purchased from E. Merck, Darmstadt.

From Sigma Chemicals Corp. were obtained NADP (Sigmagrade), glucose-6-phosphate, glucose-6-phosphate dehydrogenase type XV. All other chemicals and solvents were obtained from E. Merck, Darmstadt and were of *pro analysi* quality.

7α-Hydroxylase assay

Preparation of testicular homogenates and conditions of incubations were as previously published [3], except for incubation time which was raised from 10 to 30 min, due to the low 7α-hydroxylase activity observed in immature rats. Incubations were stopped by addition of 2 ml ice cold ethyl acetate containing nonradioactive steroids (15 μg/ml) each of testosterone, 4-androstene-3,17-dione, 5α-androstane-3,17-dione, Dht, androsterone, epiandrosterone, 3α-A'DIOL, 3β-A'DIOL, 7α-hydroxy-testosterone, 7α-hydroxy-Dht, 7α-hydroxy-3α-A'DIOL and 7α-hydroxy-3β-A'DIOL). The incubates were extracted with this solution, followed by two extractions with 2 ml ethyl acetate containing no added steroids. The combined extracts were evaporated to dryness under a stream of nitrogen. The residues were streaked on TLC plates and chromatographed as described below. Analysis of the data obtained was done as published [3]. Total 7α-hydroxylase activity was recorded as the sum of formation of the following isolated steroids: 7α-hydroxy-testosterone, 7α-hydroxy-Dht, 7α-hydroxy-3α-A'DIOL and 7α-hydroxy-3β-A'DIOL.

Isolation and identification of metabolites

Steroids were separated by TLC on silica gel-60 by one development in $CHCL_3$/acetone (6/4, v/v) [13]. Radiochemical purity of compounds thus isolated could be confirmed by recrystallization to constant specific activity. Crystallizations were performed the following way: after chromatography, the steroid fractions were scraped off the silica gel plate, extracted with ethyl acetate and the extract evaporated to dryness. About 10 mg of authentic steroid was then added to the residue and the mixture crystallized as described [14].

RESULTS

When tritiated testosterone or tritiated 3α-A'DIOL were incubated with testicular homogenates from mature or immature rats, several tritiated 7α-hydroxylated metabolites could be isolated and identified (Tables 1, 2). The incubations were conducted in the presence of a NADPH-generating system. The identity of biosynthesized 7α-hydroxy testosterone, 7α-hydroxy-Dht, 7α-hydroxy-3α-A'DIOL and 7α-hydroxy-3β-A'DIOL were assured by crystallization to constant specific activity.

Incubating testosterone with testicular homogenates from sexually immature and mature rats gave different metabolic patterns. In the mature rat testis 7α-hydroxy-testosterone dominated as the 7α-hydroxylated metabolite, while none of the isolated 7α-hydroxylated metabolites dominated quantitatively in the immature rat testis (Table 1). Measured total 7α-hydroxylase activity in mature rat testis using testosterone as the substrate, was about six times higher than that in immature testis (Table 1). Total 7α-hydroxylase activity, measured with testosterone as the substrate, was higher than total 7α-hydroxylase activity measured with 3α-A'DIOL as the substrate (Tables 1 and 2). From incubations of 3α-A'DIOL with testicular homogenates, the following steroids could be isolated: 7α-hydroxy-Dht, 7α-hydroxy-3α-A'DIOL and 7α-hydroxy-3β-A'DIOL (Table 2). With 3α-A'DIOL as the substrate, measured total 7α-hydroxylase was about four times higher in adult testis homogenates than in homogenates from sexually immature rat testis (Table 2). Incubations with radioactive Dht or radioactive 3β-A'DIOL gave metabolic patterns similar to incubations with 3α-A'DIOL as substrate (data not shown).

DISCUSSION

From incubations of tritiated androgens with testicular homogenates in the presence of NADPH-generating system, we were able to isolate several 7α-hydroxylated metabolites (Tables 1 and 2). To the best of our knowledge this is the first demonstration on testicular formation of 7α-hydroxy-Dht, 7α-hydroxy-3α-A'DIOL and 7α-hydroxy-3β-A'DIOL. An assay of 7α-hydroxylase based also on measurements of 5α-reduced metabolites of 7α-hydroxy-testosterone, gives an estimate of 7α-hydroxylase activity 3–4 times greater than that based on measurement of 7α-hydroxy-testosterone only (Table 1). In the mature rat testis, however, the formation of 5α-reduced metabolits of 7α-hydroxy-testosterone was low compared to the production of 7α-hydroxy-testosterone (Table 1). Measurements of production of 7α-hydroxy-testosterone will therefore give an adequate estimate for 7α-hydroxylase activity in the mature rat testis. Also in our new 7α-hydroxylase assay, the difference in 7α-hydroxylase activity between sexually immature and mature rat testis is great (Tables 1 and 2), thus confirming previous reports [2, 3]. However, the production of 7α-hydroxy-3α-A'DIOL from testosterone is greater in immature rats (Table 1). Testosterone seems to be better precursor than 3α-A'DIOL for the evaluation of testicular 7α-hydroxylase in mature rat testis (Tables 1 and 2).

Incubations of Dht or 3β-A'DIOL with testicular homogenates in the presence of a NADPH-generating system, gave metabolic patterns similar to incubations with 3α-A'DIOL as substrate (results not shown). It has previously been shown that Dht and 3β-A'DIOL

Table 1. 7α-Hydroxylation of testosterone and its metabolites by testicular homogenates from adult and immature rat testis

Isolated steroids (fmol/min per mg protein)	Adult rats ($n = 3$)	Immature rats ($n = 5$)
5α-Androstan-3α,7α,17β-triol	9 ± 2	24 ± 3**
5α-Androstane-3β,7α,17β-triol	18 ± 3	21 ± 6
7α,17β-Dihydroxy-4-androsten-3-one	525 ± 87	27 ± 4
7α,17β-Dihydroxy-5α-androstan-3-one	87 ± 4	23 ± 7
Total 7α-hydroxylase activity	637 ± 138*	100 ± 20

100 ng [1α,2α(n)-^{3}H]-testosterone was incubated for 30 min at 32°C with a testicular homogenate from adult (>60 days old) or sexually immature (23 days old) rats in the presence of a NADPH-generating system. Values are given as mean ± SD. Total 7α-hydroxylase is calculated as the sum of 7α-hydroxylated metabolites isolated.

* Mean 7α-hydroxylase activity significantly ($P < 0.001$) greater than immature rats.

**Accumulation of 5α-androstan-3α,7α,17β-triol significantly ($P < 0.001$) greater than in mature rats.

Table 2. 7α-Hydroxylation of 5α-androstan-3α,17β-diol and its metabolites by testicular homogenates from adult and immature rat testis

Isolated steroids (fmol/min per mg protein)	Adult rats ($n = 3$)	Immature rats ($n = 4$)
5α-Androstan-3α,7α,17β-triol	224 ± 28	43 ± 7
5α-Androstane-3β,7α,17β-triol	59 ± 17	14 ± 4
7α-17β-dihydroxy-5α-androstan-3-one	5 ± 2	12 ± 7
Total 7α-hydroxylase activity	289 ± 48*	69 ± 15

100 ng [1α,2α(n)-^{3}H]-5α-androstan-3α,17β-diol was incubated for 30 min at 32°C with a testicular homogenate from adult (>60 days old) or sexually immature (23 days old) rats in the presence of NADPH-generating system. Values are given as mean ± S.D. Total 7α-hydroxylase is calculated as the sum of 7α-hydroxylated metabolites isolated.

* Mean 7α-hydroxylase activity significantly ($P < 0.001$) greater than immature rats.

are rapidly metabolized to 3α-A'DIOL when incubated with testicular homogenates in the presence of a NADPH-generating system [3, 15]. Relatively large amounts of 7α-hydroxy-3β-A'DIOL accumulated when incubating 3α-A'DIOL with testicular homogenates from immature rats. The change from a 3α-hydroxy to a 3β-hydroxy configuration most probably arise by the formation of a 3-keto group intermediate. Whether this occurs prior to or after 7α-hydroxylation is not known.

The physiological importance of testicular 7α-hydroxylase is still unknown. 7α-Hydroxylated androgens possesses no androgenic or anabolic property in bioassays [4, 5, 12]. The hormonal factors responsible for induction of testicular 7α-hydroxylase in the rat are also unknown. Our new assay for testicular 7α-hydroxylase seems to be a suitable tool for further investigation of hormonal factors responsible for augmentation of this enzymic activity in the rat testis during puberty.

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