Journal of Steroid Biochemistry, 1974, Vol. 5, pp. 577–580. Pergamon Press. Printed in Great Britain

THE ORIGIN OF 5α-ANDROSTANE-3α,17β-DIOL AND ITS 3β EPIMER IN PERIPHERAL BLOOD OF IMMATURE FEMALE RATS

BENJAMIN ECKSTEIN

Department of Zoology, The Hebrew University of Jerusalem, Jerusalem, Israel

(*Received* 15 *February* 1974)

SUMMARY

The origin of the high levels of 5α-androstane-3α,17β-diol and of 5α-androstane-3β,17β-diol found in peripheral blood of immature female rats has been investigated. These steroids were quantitated as diacetates by g.l.c. analysis on two different columns. After hydrolysis they were further identified by g.l.c. analysis as free alcohols and TMSi ethers. Castration of rats at 19 days of age lowered the level of these steroids to below 5 ng/ml blood, which was the lower sensitivity of the method used. The levels of 3α and 3β-androstanediols in blood of intact and sham-operated animals at the age of 22 days were about 150 and 100 ng/ml blood, respectively. Injections of 3α-androstanediol to rats ovariectomized at the age of 19 days resulted in the appearance of 3β-androstanediol in peripheral blood at 22 days of age. These experiments indicate the ovarian origin of 3α-androstanediol but can not exclude the ovarian origin of the 3β epimer. The significance of identifying the ovarian origin of 3α-androstanediol in the participation of the ovaries in induction of onset of puberty is discussed.

INTRODUCTION

The hormonal mechanisms involved in onset of puberty are as yet not completely understood. It is accepted that the gonads, reproductive tract, hypophysis and median eminence are capable of adult activities prior to puberty and the initiation of puberty awaits activation by appropriate stimuli (for review see [1, 2]. In a previous study we have shown that until the 25th day of age the female rat respond to systemic administration of 5α-androstane-3β,17β-diol (3β-androstanediol) by a precocious puberty: vaginal opening, ovulation and cyclicity [3]. On days 22–26 of age, high concentrations of 5α-androstane-3α,17β-diol (3α-androstanediol) and of 3β-androstanediol are present in peripheral blood of female rats [4], thus suggesting that 3β-androstanediol participates in the stimulation of the onset of puberty.

3β-Androstanediol is present in the circulation only until the 26th day of life, while 3α-androstanediol is present in peripheral blood up to the 34th day of life, and disappears after onset of puberty [4]. Recently it was shown that 3α-androstanediol inhibits postcastrational LH elevation in the immature as well as in the postpubertal rat [5], suggesting that this steroid might exert a negative feedback on gonadotrophin secretion. Since both androstanediols seem to be involved in the mechanism of onset of puberty, it was of interest to determine their origin in the immature female rat.

MATERIALS AND METHODS

Materials

All solvents and reagents were of reagent grade: Pyridine was refluxed over KOH pellets, redistilled, stored in a dark bottle and kept in a desicator. Methanol, carbon disulfide, acetone, dichloromethane and ethyl acetate were distilled through a 50 cm-long Vigreaux column. [1,2-^{3}H]-5α-androstane-3β,17β-diol (S.A. 1·1 Ci/mmol) was purchased from NEN, Boston, Mass., and purified on t.l.c. shortly before use. 5α-androstane-3α,17β-diol and 5α-androstane-3β,17β-diol were the products of Ikapharm, Ramat Gan, Israel.

Animals

Female albino rats of the Hebrew University strain (descendants of the Wistar strain) were obtained at the age of 19 days, ovariectomized or sham-operated on the same day. The rats were kept 5–6 per cage, in a temperature-controlled room under a photoperiod of 14 h light: 10 h darkness, with free access to water and to standard-pellet diet. The steroids were dissolved in olive oil and administered subcutaneously in a vol. of

SB 5/6—D

0·2 ml; control rats received 0·2 ml of oil. Blood was drawn by cardiac puncture under light ether anesthesia into a heparinized syringe.

Extraction of steroids

Blood was collected into 30 ml heparinized centrifuge-tubes, and an internal standard of 30,000 c.p.m. of [^{3}H]-3β-androstanediol in 0·1 ml of methanol was added to each test-tube for a recovery estimate. The samples were centrifuged, the plasma removed and the cells resuspended and washed with an equal volume of physiological saline. The plasma and the saline were combined, concentrated HCl (1 : 10 v/v) was added and the mixture left overnight at 4° C. This treatment leads to the hydrolysis of the conjugated androstanediols (mostly sulfates). The mixture was extracted 4 times with equal volumes of dichloromethane; the organic solvent was then washed with distilled water and evaporated. To the resulting lipid extract about 100 mg of dry silica gel G (Merck) was added to remove a viscous substance and traces of moisture and then extracted 3 times with 1 ml portions of methanol. The methanolic extract was evaporated to dryness with a stream of dry air in a conical centrifuge tube. The resulting lipid extract served for determination of steroids.

Thin layer chromatography (t.l.c.)

Plates were coated with a layer of 0·25 mm silica gel G. The silica gel from the desired zones was scraped off and eluted with methanol. Systems used in the present study were: benzene–acetone, (4:1 v/v) (system no. 1) and n-hexane–ethyl acetate, (5:1 v/v) (system no. 2).

Gas–liquid chromatography (g.l.c.)

For determination of steroids a Packard gas chromatograph model 7905 was used in conjunction with the hydrogen flame ionization detector model 809. Methyl silicone, 3% (S.E.-30) or 1% OV-225 stationary phases on Gas Chrome Q, 100–130 mesh were packed in 2 m × 4 mm glass columns. Portions (1–5 μl) of the extracts and standard steroids were dissolved in carbon disulfide and applied with a microsyringe into the column. The column was eluted with nitrogen at a rate of 40 ml/min, at a temperature of 240°C. The inlet compartment was 265°C and the detector was maintained at 260° C. Quantitation of steroids by g.l.c. (as diacetates) was carried out as described previously [6]. *Radioactivity* was measured with a TriCarb liquid scintillation spectrometer, Packard model 3003, with automatic standardization model 579. Samples for counting were dissolved in 10 ml toluene containing 0·4% 2,5-diphenyloxazole and 0·05% 1,2-di[2-(4-methyl-5-phenyloxazolyl)]-benzene.

Determination of steroids

The steroids were located on the plates by spotting the marker line with concentrated sulfuric acid, or by exposing the plate to iodine vapors. Methods used for acetylation, silylization and hydrolysis of acetates were described previously [4, 6].

RESULTS

Identification of 3α and 3β-androstanediol in peripheral blood

The steroids were identified in plasma samples obtained from 10 to 20 rats. The steroid extracts were acetylated overnight with 0·3 ml mixture of pyridine–acetic anhydride, 2:1 (v/v). After evaporation of the reagents, the residue was dissolved in methanol, applied on t.l.c. plates and developed by system no. 2. Since the two androstanediol-acetates are not separated by this system, the labeled 3β-androstanediol served as standard of recovery for both steroids. The marker, 3α + 3β-androstanediol-diacetates running parallel, were located and the eluates opposite the marker were dissolved in 0·1 ml of carbon disulfide, 5 μl of which was taken for g.l.c. analysis on both S.E.-30 and OV-225 columns. An additional aliquot was injected into the OV-225 column equipped with a stream splitter at a rate of approximately 1:9. The material emerging from the column at the place of 3β-androstanediol-diacetate was collected directly into a 30 cm-long glass capillary tube which fitted snugly into the splitter. The capillary tube was eluted with 10 ml scintillation fluid into the counting vial and the radioactivity counted for determination of recovery. The recovery in all experiments described here was 42–86%. The rest of the diacetates were hydrolyzed, the resulting alcohols applied on t.l.c. plates and developed with system no. 1. The strip of silica gel opposite 3α and 3β-androstanediols was eluted and a 1/20 aliquot taken for g.l.c. analysis on a OV-225 column. In most instances only two prominent peaks were observed, having relative retention times (5α-cholestane = 1·00) equal to the relative retention times of the authentic 3α and 3β-androstanediols. From the rest of the extracts, TMSi ethers were prepared and analyzed on a OV-225 column. Again, only two distinct peaks corresponding to the standard TMSi ethers were detected. In some cases the eluates of the 3β-androstanediol-TMSi ethers were collected and the radioactivity counted. This was regarded as an additional index of identity. The relative retention times of the different parent compounds and their derivatives are presented in Table 1.

Table 1. Relative retention times of the two parent compounds and their derivatives prepared for the identification of 5α-androstane-3α-17β-diol and its 3β epimer in peripheral blood of immature female rats. 5α-cholestane = 1·00

Derivative	S.E.-30		OV-225	
	3α	3β	3α	3β
Free form	0·36	0·36	0·49	0·55
Diacetate	0·68	0·76	0·60	0·71
TMSi ether	0·25	0·31	0·08	0·12

Table 2. Effect of ovariectomy on the level of 5α-androstane-3α,17β-diol and its 3β epimer in peripheral blood of immature rats

Experiment no.	Treatment	Blood used (ml)	3α (ng/ml)	3β (ng/ml)
1	Sham	9·6	152	114
	OVX	19·2	<5	<5
2	Sham	10·0	105	97
	OVX	22·3	<5	<5

Effect of ovariectomy on the concentration of 3α and 3β-androstanediols

As seen in Table 2 ovariectomy results in the disappearance of 3α and 3β-androstanediols from peripheral blood of immature rats. The lower limit of sensitivity of the method used for these steroids is about 10 ng, therefore, lack of a measurable response was defined as less than 5 ng/ml, when a pool of 20 ml of blood was used for the extraction and a 1/20 aliquot of the extract was injected into the column. Recovery was determined by collecting and counting the radioactivity in the material eluted from the column at the place where 3β-androstanediol was supposed to emerge, even if no peak was obtained. In intact rats and in sham operated animals the levels of 3α and 3β-androstanediols were about 150 and 100 ng/ml, respectively, which are rather high levels when compared to either the undetectable level of less than 10 pg/ml estradiol at the age of 30–33 days [7], or to the level of 1–2 ng/ml testosterone determined radioimmunologically in prepubertal male rats [8]. The concentrations of the androstanediols found here are in good agreement with those of 100 ng/ml of 3α and of 3β-androstanediols reported previously in blood of untreated rats of the same age [4].

Administration of 3α-androstanediol to ovariectomized immature rats

In incubations of immature rat ovaries with labeled pregnenolone, 3α-androstanediol was found to be the major metabolite, whereas 3β-androstanediol could not be detected ([9] and Lerner and Eckstein, (unpublished results)). This made the ovarian origin of 3β-androstanediol questionable. It was of interest therefore, to check whether 3α-androstanediol that is produced and secreted by the ovary is epimerized peripherally to 3β-androstanediol. For this purpose, rats ovariectomized at 19 days of age were injected with 100 μg of 3α-androstanediol on days 20 and 21 of age and with 50 μg on the morning of day 22. Blood was collected at noon of day 22. At this dose level and at that experimental schedule about 15% of the 3α-androstanediol was found to be epimerized outside of the ovary to 3β-androstanediol (see Table 3).

Table 3. Transformation of 3α to 3β-androstanediol in extra-ovarian tissue of immature rats. Rats were ovariectomized at the age of 19 days and injected subcutaneously with 100 μg 3α-androstanediol on days 20 and 21 and with 50 μg the morning of day 22. Blood was drawn at noon of day 22 and the levels of 3α and 3β-androstanediols determined

Experiment no.	Treatment	Blood used (ml)	3α (ng/ml)	3β (ng/ml)
1	Sham	10·5	133	80
	OVX	22·0	<5	<5
	OVX + 3α	21·5	630	95
2	Sham	12·0	202	91
	OVX	22·5	15	<5
	OVX + 3α	23·0	123	18

3α = 5α-Androstane-3α,17β-diol; 3β = 5α-androstane-3β,17β-diol.

DISCUSSION

The identification of 3α and 3β-androstanediols in peripheral circulation of immature female rats was based on two different t.l.c. systems, two g.l.c. columns for their diacetates and on g.l.c. analysis of the free alcohols and their TMSi ethers. The androstanediols were isolated and quantitated from the lipid extracts as diacetates because by acetylation of the whole lipid extract a better purification of these steroids was achieved on t.l.c., and because their molar response in this form is larger than that of their free alcohols or their TMSi ethers.

The *in vitro* production of 3α-androstanediol by homogenates from immature rat ovaries is well established [6, 9, 10]. On the other hand, 3β-androstanediol could not be detected in these incubations (Lerner and Eckstein, unpublished results). It was shown in our laboratory that incubation of 3α-androstanediol with ovarian homogenate of immature rats treated with FSH resulted in the formation of 3β-androstanediol. This reaction is catalyzed by an epimerization system that is inducible specifically by FSH [6]. Recently it

was shown that a very high concentration of estradiol-17β (measured radioimmunologically) and a high level of FSH are present in plasma of female rats until the 21st day of age [11, 12]. This together with the FSH-induced epimerase system could explain the high level of 3β-androstanediol found in blood of rats 22–26 days of age.

Table 3 shows that only about 15% of the injected 3α-androstanediol is epimerized peripherally to 3β-androstanediol. This cannot account for the endogenous ratios of 3α/3β of 0·9 to 2·4 found to be present at days 22 to 26 of age [4]. Thus it seems that 3β-androstanediol is produced by the ovary in addition to the limited quantity produced by peripheral epimerization of 3α-androstanediol. The present results together with the *in vitro* studies establish that 3α-androstanediol is produced by immature rat ovaries, but there remain reservations as for the ovarian origin of 3β-androstanediol. Kinetic experiments including metabolic clearance rate determination are needed to establish the relations between the two androstanediols.

Maturation at the ovarian level has been reported by Rubin *et al.* [13], who observed a sharp rise in ovarian delta-5-3β-hydroxysteroid dehydrogenase activity at about the time of the first ovulation. From the proximity of this change to the first ovulation, it is obvious that it does not necessarily initiate onset of puberty. By establishing the ovarian origin of 3α-androstanediol, an early maturational change in ovarian steroidogenesis has been documented. This finding justifies the assumption that ovaries participate in the initiation of the onset of puberty, an assumption which is in contrast with earlier observations that had excluded the ovaries as the organ responsible for the normal onset of puberty [14, 15].

Acknowledgements—The author wishes to thank Miss Sulamith Yehud for skilled assistance and Dr. J. Shani for valuable assistance in preparing the manuscript. This work was supported by grants No. M 73.151 from the Population Council and from the Central Research Fund of the Hebrew University.

REFERENCES

1. Donovan B. T. and van der Werff ten Bosch J. J.: In *Physiology of Puberty*, Williams and Wilkins Co. Baltimore, 1965.
2. Critchlow V. and Bar Sela M. E.: In *Neuroendocrinology*, (Edited by Martini L. and Ganony W. P.) vol. II. Academic Press, New York, 1967, 101–162.
3. Eckstein B., Golan R. and Shani (Mishkinsky) J.: *Endocrinology* **92** (1973) 941–945.
4. Eckstein B. and Ravid R.: *Endocrinology* **94** (1974) 224–229.
5. Eckstein B., Yehud S., Shani (Mishkinsky) J. and Goldhaber G.: in preparation.
6. Eckstein B. and Springer C.: *Endocrinology* **89** (1971) 347–352.
7. Endersby C. A., Gallagher M., Horton C. E., McDonald P. G. and Wilson C.: *J. Endocr.* **57** (1973) lx–lxi.
8. Swerdloff R. S., Walsh P. C., Jacobs H. S. and Odell W. O.: *Endocrinology* **88** (1971) 120–128.
9. Springer C. and Eckstein B.: *J. Endocr.* **50** (1971) 431–439.
10. Mason N. R.: *Endocrinology* **87** (1970) 350–355.
11. Weisz J. and Gunsalus P.: *Endocrinology* **93** (1973) 1057–1065.
12. Meijs-Roelofs H. M. A., Uilenbroek J. Th. J., de Jong F. H. and Welschen R.: *J. Endocr.* **59** (1973) 295–304.
13. Rubin B. L., Deane H. W., Hamilton J. A. and Driks E. C.: *Endocrinology* **72** (1963) 924–930.
14. Foa C.: *Archs Ital. Biol.* **34** (1900) 43–73.
15. Long J. A. and Evans H. M.: *Mem. Univ. Calif.* **6** (1922) 1–148.