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IN VITRO OESTROGEN BIOSYNTHESIS BY HUMAN POLYCYSTIC OVARIES

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SUMMARY

In vitro steroid biogenesis by human polycystic ovaries has been investigated in four patients. Aliquots from each ovarian homogenate were incubated *in vitro* with tritiated pregnenolone, dehydroepiandrosterone and androstenedione. Labeled metabolites were isolated and identified by crystallizations to constant S.A.

The general pattern of steroid metabolism in those four cases is very similar to that reported from incubations of normal ovarian tissue. It should be noticed particularly that significant amounts of oestrone and oestradiol were recovered from each incubation, whatever the substrate. This finding does not match with a lack of aromatization, therefore it is suggested that such enzyme deficiencies in the polycystic ovaries are not so common as it is generally assumed in the literature.

A ratio of oestrone to oestradiol lower than one was observed in most of the polycystic ovary incubations, whereas in normal ovaries that ratio is usualy higher than one. That trend is the only descrepancy that is suggested by the comparison between normal and polycystic ovaries. However, such difference could be the result rather than the origin of the menstrual cycle disturbance.

INTRODUCTION

The endocrine disorders associated with polycystic ovarian disease are characterized by an increase of serum LH with subnormal FSH, persistenly low estradiol and progesterone and a high level of serum testosterone. That peculiar picture can be observed in patients consulting for secondary amenorrhea, obesity, sterility and hirsutism, and presenting micropolycystic ovaries. It is assumed that the feed-back mechanism controlling the menstrual cycle is impaired. However, the origin of this disturbance remains doubtful.

Enzymatic defects of the ovarian steroidogenesis, similar to those observed in the congenital adrenal hyperplasia, has been proposed as an "explanation" for the hyperandrogenia [1]. Mahesh and Greenblatt[2] have suggested a defect of the 3β-hydroxysteroid dehydrogenase, but such a metabolic block should induce a decrease rather than an increase of serum testosterone [3]. For Axelrod and Goldzieher[4] the accumulation of C-19 metabolites and the lack of estrogens arises from a deficiency of the aromatizing capacity. However the estrogen level measured in the serum or the urine from these patients can hardly be explained only by the peripheric aromatization of neutral steroids [1, 2].

The present investigation was undertaken with the intention to trace the pattern of estrogen biogenesis by the polycystic ovary *in vitro*.

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EXPERIMENTAL

Clinical material. Ovarian tissue was obtained from five patients, four of them presenting clinical and biological evidence of "Stein Leventhal syndrome" (spaniomenorrhoea, obesity, hirsutism, increased basal level of serum LH with a normal FSH and elevated testosterone). This was confirmed by the typical aspect of micropolycystic ovaries. The last patient was a normal woman with regular ovulatory cycles.

Trivial names. Androstenedione (4-androstene-3, 17-dione), dehydroepiandrosterone (3β-hydroxy-5-androstene-17-one), 17α-hydroxyprogesterone (17α-hydroxy-4-pregnene-3, 20-dione), oestradiol (1, 3, 5 (10)-oestratriene-3, 17β-diol), oestriol (1, 3, 5 (10)-oestratriene-3, 16α, 17β-triol), oestrone (3-hydroxy-1, 3, 5 (10)-oestratrien-17-one), pregnenolone (3β-hydroxy-5-pregnen-20-one), progesterone (4-pregnene-3, 20-dione) and testosterone (17β-hydroxy-4-androsten-3-one).

Labeled steroids. The following labeled steroids were incubated *in vitro*: [7-^{3}H]-androstenedione TRA-164* (S.A. 10.6 Ci/mmol), [7-^{3}H]-dehydroepiandrosterone NET-033† (S.A. 14.4 Ci/mmol) and [7-^{3}H]-pregnenolone NET-039† (S.A. 19.8 Ci/mmol). Tracer amounts of [4-^{14}C] steroids† were used for the detection of metabolites on chromatograms: androstenedione NEC-136, dehydroepiandrosterone NEC-206, oestradiol NEC-127, oestriol NEC-565, oestrone NEC-512, progesterone NEC-081 and testosterone NEC-101. Prior to incubation, 10 μCi of each tritiated labeled steroid, in benzene–ethanol mixture, were added to separated incubation vials, after evap-

Table 1. Identified material, expressed as percent of incubated material and corrected for methodological losses

Experiment	Polycystic ovaries 1			2			3			4		Normal ovary 5	
Incubated steroid	Δ_5P‡	DHA	A	Δ_5P‡	DHA	A	Δ_5P‡	DHA	A	Δ_5P‡	A	DHA	A
Oestrone (OE_1)	1.81	9.73	5.93	0.23	0.86	0.89	0.63	2.42	1.30	0.12	2.21	0.71	7.50
Oestradiol (OE_2)	5.25	13.39	7.58	0.32	1.69	1.89	1.94	4.17	3.86	0.11	1.57	0.50	0.68
OE_1/OE_2	0.34	0.73	0.78	0.72	0.51	0.47	0.33	0.58	0.34	1.09	1.41	1.42	11.03
Androstenedione (A)	4.77	12.20		12.90	27.38		17.84	32.49		1.24		0.87	
Testosterone (T)	(1.97)*	0.89	1.48	(0.68)*	3.09	3.65	(0.46)*	(0.09)*	2.30	(0.18)*	2.89		
A/T	2.42	13.71		18.97	8.86		38.78	361		6.89			
Dehydroepiandrosterone (DHA)	—†			—†			—†			4.60			
Progesterone	7.66			(0.44)*			(0.61)*			1.15			
17α-Hydroprogesterone	(0.02)*												

* Values in brakets represent an estimation when constant SA could not be achieved. Ratios estrone/estradiol and androstenedione/testosterone are also represented. † Undetectable. ‡ Pregnenolone.

oration to dryness, the radioactive material was dissolved in 100 μl of absolute ethanol.

Measurement of radioactivity. Tritium and carbon fourteen were measured simultaneously with a three-channels liquid scintillation spectrometer Packard* model 3380. Water soluble radioactive material was counted in Instagel* and toluene soluble radioactivity in Permafluor*–toluene mixture (1:25, v/v). For the determination of S.A., crystals and mother liquors (approximatively 1 mg) were dissolved in 0.5 ml of ethanol prior to the addition of scintillation mixture.

Incubation. Wedge resection of both ovaries was performed at laparotomy. A slice was cut off for pathology and the remaining tissue (4.4–6.6 g) was combined for incubation. This material was homogenized with an Ultra-Turax mixer in six vols of buffer (0.05 mol phosphate buffer with 0.25 mol sucrose and 0.04 mol nicotinamide). The homogenate was then divided in three aliquots, each of them was transferred in an incubation vial containing one of the tritium labeled steroids. Incubations were run at 37°C with air as the gas phase, in a Dubnoff incubator. Ten mmol of ATP and NADP were added to each vial at the beginning of the incubation. After four h, incubations were stopped by addition of four vols of ethanol and the samples were stored at −32°C until processing.

Extraction and isolation of metabolites. After centrifugation, the supernatant was collected, evaporated to dryness and submitted to a partition between pentane and 90% methanol. Pentane was discarded and after evaporation of the methanol, the ether soluble (unconjugated) and water soluble (conjugated) radioactive material was separated according to Mikhail *et al.*[5]. The unconjugated fraction was submitted to a phenolic partition with back wash using 1N NaOH and toluene. The NaOH fraction was taken to pH 8.5 with 6N H_2SO_4, and steroids were re-extracted by ether.

Identification of metabolites. After further purifications by chromatography (see below), final identification was achieved by addition of the appropriate carrier and crystallization to constant S.A. Usually the material was acetylated prior to the third crystallization. Conversion rates were calculated on the basis of the mean value of the two last crystals and mother liquors.

RESULTS

1. *Unconjugated material and phenolic partition*

After the ether–water partition some 5% of the radioactivity remained in the water phase (1.6–13.8%), this material was not analyzed. In the unconjugated (ether soluble) fraction, the phenolic material ranged from 2.8 to 43%, with a mean value of 11%. Only a part of this phenolic-like material could be identified as oestrone and oestradiol.

2. *Isolation and identification of oestrogens*

The metabolites present in the phenolic fraction were separated by chromatography on celite column according to Siiteri[6] using the system *n*-hexane–ethyl acetate–methanol–water (17:3:7:3, by vol.). Oestrone, and oestradiol were collected separately, mixed with tracer amounts of the appropriate [4-^{14}C]-oestrogen and purified by chromatography on Sephadex LH-20† in benzene-methanol (85:15, v/v) (Carr *et al.*[7]) prior to crystallization. As shown on the table, aromatization of neutral precursors into oestrone and oestradiol occurred in all incubations. Each individual ovary shows roughly the same conversion rate whatever the substrate, but large variations appear from one ovary to the other. The ratio oestrone/oestradiol from polycystic ovary incubations is usualy lower than one while that from the normal ovary is higher.

3. *Isolation and identification of neutral metabolites*

(*a*) *From androstenedione.* The neutral fraction was mixed with tracer amount of [4-^{14}C]-testosterone, purified by chromatography on Sephadex LH-20 (*n*-hexane–benzene–methanol 80:15:5, by vol.) and crystallized to constant S.A.

(*b*) *From dehydroepiandrosterone.* After addition of [4-^{14}C]-androstenedione and testosterone the neutral

*Packard Instrument Company Inc., Downers Grove, IL 60515, U.S.A.

†From Pharmacia, Uppsala, Sweden.

fraction was submitted to a first chromatography on Sephadex LH-20 (*n*-hexane–benzene–methanol 80:15:5, by vol.). In this system, dehydroepiandrosterone and androstenedione have a similar mobility but separation could be achieved by acetylation followed by chromatography in the same system.

(*c*) *From pregnenolone.* After addition of [4-^{14}C]-tracers, progesterone, androstenedione, dehydroepiandrosterone and testosterone were separated by chromatography on Sephadex LH-20 (*n*-hexane–benzene–methanol 80:15:5, by vol.). As described previously the separation of androstenedione from dehydroepiandrosterone was obtained by acetylation and chromatography in the same system. The conversion rates from the various incubations are presented in the table. Testosterone and androstenedione were identified in all incubations. The ratio androstenedione/testosterone was higher than 1 except in experiment 4. Conversion of pregnenolone to dehydroepiandrosterone occurred only in one out of four polycystic ovaries. Progesterone was isolated from the four pregnenolone incubations, but in two of them lack of radioactive material precluded to final identification. 17α-Hydroxyprogesterone was detected only in the experiment that presented the higher conversion rate of pregnenolone into progesterone. Here also constant S.A. could not be achieved after six crystallizations.

COMMENTS

Most of the patients with polycystic ovaries presented an abnormal hormonal pattern, characterized by an elevation of circulating androgens, especially testosterone (Easterling *et al.*[3]). It has been suggested that the ovarian steroidogenesis could be modified by the deficiency of some enzyme systems. Indeed, results from *in vitro* incubations have shown in some patients a lack of aromatization of neutral steroids into oestrogens with an accumulation of C-19 metabolites [4, 8]. This is in agreement with the presence of high levels of androgens in the fluid from these ovarian micro cyst [9]. However, in the present investigation, oestrone and oestradiol have been identified from all incubations, whatever the substrate. That finding rules out the hypothesis of a total defect of the aromatization capacity [4, 8–10]. A deficiency at the level of the 3β-hydroxysteroid dehydrogenase and isomerase has also been put forward as an alternate metabolic block [2]. Such enzyme deficiency seems unlikely in the present cases since an extensive conversion of 3β-hydroxy-5-ene-steroids into 3-oxo-4-ene-steroids occurred in three experiments, while in the last incubation, that conversion, although lower, was undoubtly effective.

The only feature that could make the difference between normal and abnormal ovaries is the oestrone/oestradiol and the androstenedione/testosterone ratio. It seems, in comparison to data reported elsewhere from normal ovary incubations, that in the present experiment the equilibrium for polycystic ovary is shifted in favor of the 17β-hydroxyl group for oestrogens but not for the C-19 steroids. This is only a speculative proposition that needs more experiments to be confirmed. The polycystic ovary is presumed to secrete more androgens than oestrogens, however, surprisingly, the results from the *in vitro* incubations indicate a trend to produce more oestradiol, the biologically active oestrogen, than oestrone, but less testosterone, also the biologically active hormone, than androstenedione. That would suggest that the steroid pattern observed in the polycystic ovarian disease is the result of a disturbance of the secretion rather than an abnormal biosynthesis. However, the interpretation of data from *in vitro* experiments should be very cautious, and it is difficult to determine if the picture obtained from polycystic ovaries incubations really reflects abnormalities of steroidogenesis that exist *in vivo*, or are related to the experimental design. Among the various parameters that could modify the steroid metabolism *in vitro*, the S.A. of the labeled steroid is seldom taken in consideration, although the use of carbon fourteen labeled hormone may represent a few hundreds μg of steroid dissolved in a very small volume, raising the concentration to levels never met *in vivo* that could induce an inhibition the enzymic process.

The result from the present investigation do not bring any valuable argument to support the hypothesis of an abnormal ovarian steroidogenesis resulting from a primary enzyme defect. The Stein Leventhal syndrom is certainly not a single entity and the disturbances of steroidogenesis could be in some cases, the "*primum movens*" of the abnormal ovarian secretion, however it is more likely that, at least at the beginning, the ovarian function is normal and that the morphological and functional alterations are related to a primary disturbance of the hypothalamo-pituitary control of the menstrual cycle. Indeed this syndrome usually occurs after a period of normal cycles, moreover ovulation and normal steroidogenesis may be resumed after wedge resection or Clomiphene administration and it is difficult to understand how such treatment could induce directly the formation of a deficient enzyme.

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