COMPARATIVE STUDIES OF ANDROGEN METABOLISM IN THECA AND GRANULOSA CELLS OF HUMAN FOLLICLES IN VITRO

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Received 1-8-82

ABSTRACT

In eight separate experiments, theca and granulosa were isolated from human follicles (5-25 mm in diameter), and their capacities to metabolize radiolabelled testosterone in 24 hour cultures were assessed. Theca metabolized testosterone primarily to androstenedione, however significant aromatization to estradiol-17β and to estrone was also observed. Granulosa metabolized testosterone primarily to estradiol-17β and estrone, while smaller quantities were converted to androstenedione. In seven of these experiments, the intermediate of aromatization, 19-hydroxytestosterone, was identified. In six of these experiments, theca, when compared to granulosa, produced more androstenedione but less estradiol-17β and estrone. 5α-Reduced androgens were non-detectable or produced in small quantities. In a single experiment, metabolism of androstenedione was compared to metabolism of testosterone by both theca and granulosa. Theca metabolized androstenedione to testosterone in smaller quantities than testosterone to androstenedione. Granulosa metabolized androstenedione to testosterone in higher quantities than testosterone to androstenedione. Both theca and granulosa aromatized androstenedione more readily than testosterone.

INTRODUCTION

Recent evidence indicates that androgen metabolism may play a key role in steroidogenesis by human ovaries. Testosterone and androstenedione are known to undergo aromatization to estrogens. Both aromatizable and nonaromatizable androgens have been shown to stimulate progesterone production by human granulosa cells (1), as well as increase FSH induction of aromatization, presumably via androgen receptors (2). Non-aromatizable androgens are also reported to act as inhibitors of aromatization (3). Our previous studies on human follicles have demonstrated that aromatizable androgens are produced

predominantly by theca (4), while aromatization occurs predominantly in granulosa (5). Further, we demonstrated that granulosa cells metabolize radiolabelled testosterone mainly into estrogens in vitro (6). The purpose of the present study is to compare the ability of human theca and granulosa cells to metabolize androgens in culture. We isolated theca and granulosa of the same follicle and the ability of these two compartments to metabolize radiolabelled testosterone and androstenedione was assessed.

MATERIAL AND METHODS

Human ovarian follicles were obtained from women undergoing ovariectomy for various gynaecological conditions. The clinical data of these patients are summarized in Table 1. These patients were at various stages of the menstrual cycle and had received no preoperative treatment. Nonatretic follicles characterized by a bright, translucent appearance with extensive vascularization but containing no blood clots, as determined by inspection under the dissecting microscope, were dissected intact. Theca and granulosa cells were isolated as described previously (4,5). The medium used was Eagle's minimum essential medium, supplemented with L-glutamine (4mM), non-essential amino acids (0.1 mM), penicillin (100 units/ml), streptomycin (100 µg/ml), fungizone (250 ng/ml) and 5 percent heat inactivated fetal calf serum (GIBCO). Isolated theca and granulosa cells were washed twice with the medium, and incubated at 36°C under an atmosphere of 5 percent CO_2 in air. Testosterone-4-^{14}C at a concentration of 10^{-6} M (specific activity 51.9 mCi/mmol, New England Nuclear Corp.) was added to all cultures for 24 hours. Impurity and decomposition products of labelled testosterone, not associated with the metabolism of tissue studied, were assessed by incubating testosterone-4-^{14}C in the medium alone. At the end of incubation, the media were harvested, centrifuged at 300 x g for 5 minutes and frozen at -24°C until subsequent analysis. Cells were washed twice with 0.9 percent NaCl and proteins were assayed by a modified Lowry method (7). The culture media were extracted with five volumes of ethyl ether; extracts were evaporated under nitrogen flow in a 35°C water bath and reconstituted in 100 µl of absolute ethanol containing the following carrier steroids: 0.5-1 µg each of testosterone (17β-hydroxy-4-androsten-3-one, T), androstenedione (4-androstene-3,17- dione, A), dihydrotestosterone (17β-hydroxy-5α-androstan-3-one, DHT), 19-hydroxytestosterone (17β, 19-dihydroxy-4-androsten-3-one, 19-OH T), 19-hydroxyandrostenedione (19-hydroxy-4-androstene-3,17-dione, 19-OH A), 5α-androstane-3α,17β-diol (3α-diol), 5α-androstane-3,17-dione (androstanedione, A-dione), estradiol-17β (1,3,5(10)-estratriene-3,17β-diol, E_2), estrone (3-hydroxy-1,3,5(10)-estratrien-17-one, E_1) and estriol (1,3,5(10)-estratriene-3,16α,17β-triol, E_3). 19-Hydroxytestosterone was

obtained from Steraloids; all other steroids were purchased from Sigma. Each extract was applied to a silica gel thin layer plate (Eastman) and then developed in a system of benzene-ethyl acetate (2:1, vol/vol). Radioactive metabolites were detected by radioautography by exposure of chromatograms to no-screen medical X-ray film (Kodak). Subsequently, the chromatograms were sprayed with sulfuric acid-ethanol (1:1, vol/vol) and charred for 15 minutes at 120°C in order to visualize the carrier steroids. The radioactive steroids were identified on a preliminary basis by aligning and transilluminating the chromatograms with their corresponding radioautograms. Radioactive zones were cut out and radioassayed in a LS-9000 liquid scintillation counting system (Beckman). Some chromatograms were not subjected to charring. Radioactive steroids were eluted and identified by recrystallization to constant specific activity following addition of carrier steroids (8). In a single experiment, a comparative study of the metabolism of testosterone and androstenedione by theca and granulosa was carried out. Androstenedione-4-^{14}C at a concentration of 10^{-6} M (specific activity 57.4 mCi/mmol, New England Nuclear Corp.) was employed in a manner identical to testosterone, as described above.

TABLE I
SUMMARY OF PATIENTS AND FOLLICLES STUDIED

Patient	Experiment Number	Age (yrs)	LMP[a]	Number and Size (mm in diameter) of Follicles Studied
A	1	33	14	1 x 5
B	2	45	2	1 x 10
	3			1 x 8
C	4	36	-[b]	1 x 15
D	5	29	-[c]	1 x 7 and 1 x 8
	6			1 x 25
E	7	51	28	1 x 8 and 1 x 10
F	8	44	15	1 x 15

a = days from the onset of last menstrual period
b = no data available
c = patient had hysterectomy two years prior to experiment

RESULTS

Major Metabolites of Testosterone and their Identification

Human theca and granulosa metabolized testosterone-4-^{14}C in all

eight experiments. The recovery of the incubated radioactivity in the medium varied from 75 to 85 percent. Testosterone was converted to several steroids. Seven products, contributing to more than 98 percent of the extracted metabolites of testosterone, were identified on the basis of mobility on thin layer chromatography (TLC) plates: androstenedione, estradiol-17β, estrone, 19-hydroxytestosterone, dihydrotestosterone, androstanedione and 5α-androstane-3α,17β-diol. Radiochemical purity of the recovery substrate (testosterone) and five of its metabolites - androstenedione, estradiol-17β, estrone, 19-hydroxytestosterone and dihydrotestosterone - was confirmed by recrystallization to constant specific activity, as presented in Table II. Androstanedione and 5α-androstane-3α,17β-diol were produced in minute quantities and their recrystallization was not carried out. The histograms presenting experimental data of conversion of testosterone to seven identified steroids by human theca and granulosa are shown in Figure 1.

Profile of Metabolism of Testosterone by Theca

Theca readily converted testosterone to androstenedione in all eight experiments. In six experiments (#1-5 and 8), androstenedione was the major product ranging from 58.6 to 89.7 percent of all seven observed metabolites. The second most abundant product was estradiol-17β, constituting 1.7-15.6 percent of all metabolites studied. In the remaining two experiments (#6 and 7), estradiol-17β was the major metabolite contributing 60.9 and 63.7 percent, while conversion to androstenedione was 15.0 and 22.1 percent. In all experiments, the third major product of testosterone was estrone, with levels of 0.9-19.6 percent of all observed metabolites. The levels of 19-hydroxy-

testosterone, the intermediate of the aromatization reaction of testosterone, were low and ranged from 0 (undetectable) to 4.4 percent. The intermediate of aromatization of androstenedione, 19-hydroxyandrostenedione, was undetectable in all experiments. Production of 5α-reduced androgens by theca was consistently low or undetectable.

TABLE II
RADIOCHEMICAL IDENTIFICATION OF METABOLITES OF TESTOSTERONE-4-^{14}C BY RECRYSTALLIZATION

Compound	Carrier (mg)	Recrystallized from	Specific activity (dpm/mg) after recrystallization number 0	1	2	3	4
E_2	59.6	acetone/hexane	339	336	320	347	345
E_1	10.5	acetone/hexane	471	558	559	543	558
19-OH T	6.2	methanol:chloroform/hexane	547	561	572	543	568
A	59.4	acetone/hexane	609	620	619	632	621
T	42.6	acetone/hexane	1,210	1,210	1,250	1,220	1,240
DHT	24.5	acetone/hexane	46	44	52	-	-

Profile of Metabolism of Testosterone by Granulosa

Estradiol-17β and estrone were the major metabolites of testosterone in seven experiments (#2-8), ranging from 34.7 - 84.5 percent and 5.1 - 23 percent of all metabolites studied, respectively. In one experiment (#1) the major metabolite was androstenedione, 52.9 percent, followed by estradiol-17β, 22.6 percent, and estrone, 7.3 percent. 19-Hydroxytestosterone was detected in seven experiments (#1,2 and 4-8) with levels of 2 - 15.5 percent, while 19-hydroxyandrostenedione was undetectable in all experiments. 5α-Reduced androgens were undetectable or produced in small quantities.

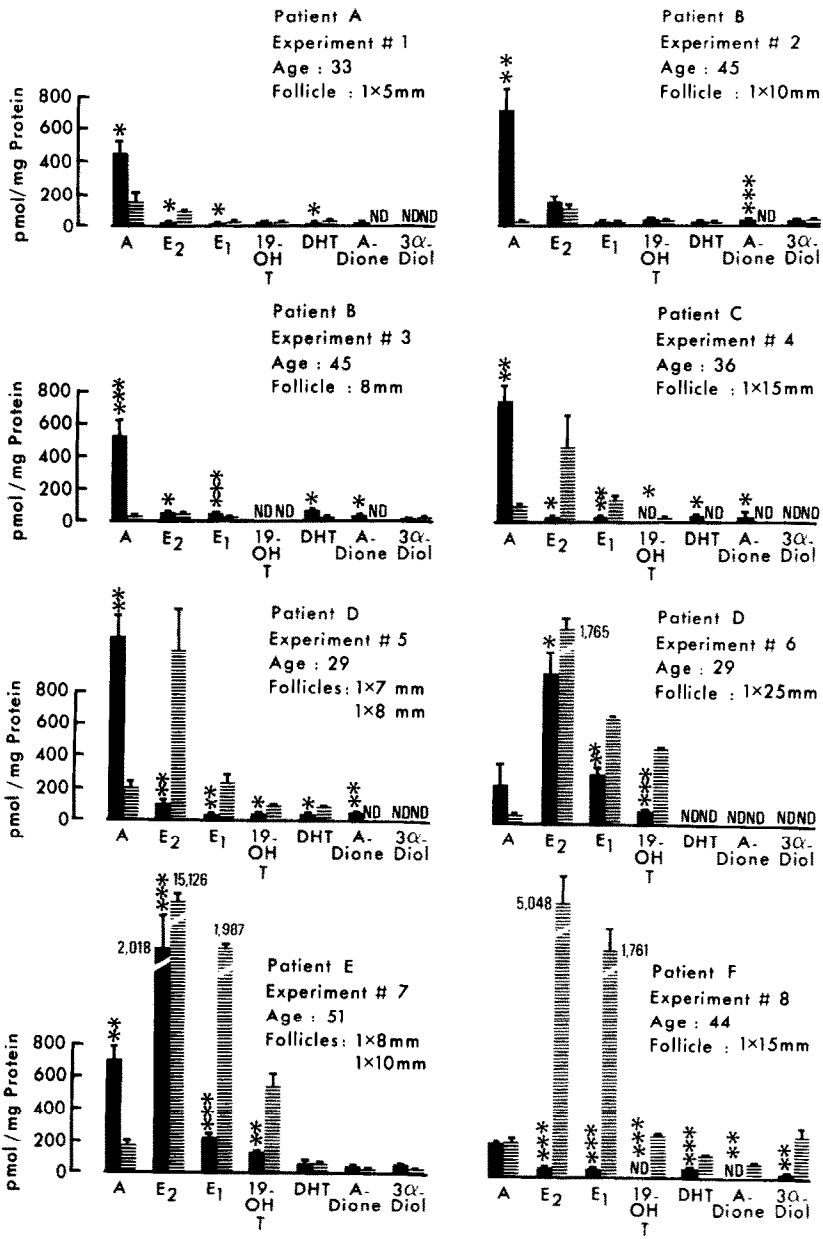

Fig. 1: Conversion of testosterone-4-^{14}C by theca and granulosa cells during 24 hr incubation (Mean ± SEM); solid bars, theca; hatched bars, granulosa; ND: non-detectable. Data were analysed by Student's t test; *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$

Comparison of Metabolism of Testosterone by Theca and Granulosa

In the experiments conducted there was a high variability of steroidogenic capacities in theca and granulosa components from different patients. However, when comparing components from the same follicles, certain trends were observed. Theca showed significantly higher conversion of testosterone to androstenedione than granulosa in six experiments (#1-5 and 7). In the remaining two experiments (#6 and 8) no significant difference was observed. Granulosa showed significantly greater aromatization of testosterone to estradiol-17β and to estrone than theca in six experiments (#1 and 4-8). In experiment #2, no statistical difference was observed, while in experiment #3, theca accumulated significantly more estradiol-17β and estrone than its granulosa counterpart. 19-Hydroxytestosterone, the intermediate of aromatization, accumulated in significantly higher amounts in granulosa than in theca cultures in five experiments (#4-8), while in remaining experiments (#1-3) no statistical difference was observed. No consistent patterns in the distribution of 5α-reduced metabolites between theca and granulosa components could be determined.

Comparison of Metabolism of Androstenedione-4-^{14}C and Testosterone-4-^{14}C

In a single experiment, equal amounts of radiolabelled androstenedione and, in parallel incubation, of radiolabelled testosterone, were cultivated with human theca and granulosa. Results are presented in Table III.

Androstenedione was converted to the same identifiable products as testosterone; however the yield of products was different. In theca, conversion of androstenedione to testosterone was lower than conversion

of testosterone to androstenedione ($p < 0.05$).

TABLE III
COMPARISON OF METABOLISM OF ANDROSTENEDIONE AND TESTOSTERONE BY HUMAN THECA AND GRANULOSA[a]

	THECA		GRANULOSA	
Metabolite	SUBSTRATE Androstenedione	Testosterone	SUBSTRATE Androstenedione	Testosterone
T	257 ± 49	-	327 ± 26	-
A	-	699 ± 65	-	164 ± 16
E_2	2,740 ± 96	2,018 ± 207	17,975 ± 518	15,126 ± 28
E_1	236 ± 51	209 ± 32	1,883 ± 45	1,987 ± 13
19-OH T	30 ± 6	105 ± 5	212 ± 24	541 ± 59
DHT	ND	55 ± 15	ND	54 ± 10
A-dione	50 ± 15	31 ± 4	64 ± 10	15 ± 3
3α-diol	50 ± 22	54 ± 24	103 ± 3	21 ± 4

ND - non-detectable
a - Theca and granulosa were obtained from patient E
Follicles were 8 and 10 mm in diameter
Values are in pmol/mg protein and represent means ± SEM

The opposite pattern was observed for granulosa, where conversion of androstenedione to testosterone was greater ($p < 0.05$). Both theca and granulosa produced more estradiol-17β from androstenedione than from testosterone ($p < 0.05$). Accumulation of estrone within either theca or granulosa cultures was not significantly altered when androstenedione was used as the substrate instead of testosterone. When androstenedione rather than testosterone was used, both theca and granulosa cultures produced significantly lower quantities of 19-hydroxytestosterone ($p < 0.01$ and $p < 0.05$, respectively). In theca, both substrates gave a similar yield for 5α-reduced androgens. In granulosa, conversion

to androstanedione and 5α-androstane-3α,17β-diol was higher from androstenedione than testosterone ($p < 0.01$). Conversion of androstenedione to dihydrotestosterone was undetectable.

DISCUSSION

The present experiments provide a direct comparison of the enzymatic systems of human theca and granulosa involved in the metabolism of testosterone. The enzymatic activities monitored in this study were 17β-hydroxysteroid dehydrogenase, aromatase and 5α-reductase. It is necessary to point out that figures of enzymatic activities of theca were most probably under-estimated in this study due to the presence of endogenous androgens (4) which could dilute the labelled substrate, testosterone. This was unlikely to be the case with regard to granulosa cells which have been shown to lack or have low levels of the 17α-hydroxylase and/or $C_{17,20}$-lyase enzymes necessary to synthesize androgens as shown in rats (9) and humans (4). However, it seems unlikely that the effect of dilution of the radiolabelled substrate could interfere with the observations of relative activities of the enzymatic systems studied.

The results obtained, although variation existed between individual follicles, showed several differences in patterns of metabolism of testosterone by theca versus granulosa. Theca had a very active 17β-hydroxysteroid dehydrogenase system, significantly more active than granulosa. In most cases, this activity was higher than aromatase activity and in all cases higher than 5α-reductase activity. Since it has been found that androstenedione is the major androgen secreted by the human ovary (10) and by theca in particular (11,12), it is likely that 17β-hydroxysteroid dehydrogenase of thecal origin is

at least in part responsible for predominant production of androstenedione over testosterone. Our data of comparative metabolism of androstenedione and testosterone indicate that, in theca, the equilibrium of the reaction, testosterone↔androstenedione is shifted toward the latter compound. In granulosa, the equilibrium seems to be shifted toward testosterone. Results of the same experiment suggest also that androstenedione is a better substrate for aromatization than testosterone in both theca and granulosa. It would appear that 17β-hydroxysteroid dehydrogenase and aromatase of both thecal and granulosa origin cooperate in production of estradiol-17β. It is of interest to note that results of the studies of rat ovarian tissues suggest that these enzymatic activities are regulated by gonadotropins in a similar manner (13,14,15).

In our experiments we were able to demonstrate consistently the presence of aromatase activity in both granulosa and theca cultures. The possibility that estrogen production by theca cultures may be due to contamination by granulosa cells seems unlikely, since the isolation procedures, as described previously (5), allow separation of theca components free of histologically detectable granulosa cells. It is suggested that granulosa is the major cellular site of estrogen production in humans, as observed previously (5,16), although in some follicles theca may also significantly contribute to total follicular output of estradiol and estrone. This suggestion is agreeable with the findings of McNatty et al. (12,17) that all steroid secreting tissues in ovary have the capability of producing estrogens. The present studies indicate that the enzymatic systems of theca and granulosa cells differ quantitatively rather than qualitatively. The variabil-

ity of aromatase activity observed in theca cultures may reflect differences in degree of follicular maturation, as has been previously reported for granulosa cells (3). However, the clinical data available was insufficient to draw any firm conclusions on this point.

It is of interest to find significant amounts of 19-hydroxytestosterone in some of the granulosa cultures in the present study. This finding is in accord with suggestions of Dorrington and Armstrong (18) that the sequence of reactions in aromatization involves the formation of 19-hydroxylated intermediates. Moreover, the presence of "free" (secreted to the incubation medium) intermediate suggests that 19-hydroxytestosterone is actually a discrete compound and does not only represent an enzyme-bound transition state of conversion of testosterone to estradiol.

ACKNOWLEDGEMENTS

This research was supported by grants from the Medical Research Council of Canada (MA-7298) and from the B.C. Health Care Research Foundation (65-3956). We would like to thank Dr. Gary Jackson for providing the specimens.

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