Steroid production from 17 α -hydroxypregnenolone and dehydroepiandrosterone by human granulosa cells *in vitro*

Ruth E. Fowler, N. L. Fox, R. G. Edwards and P. C. Steptoe*

*Physiological Laboratory, University of Cambridge, and *Oldham and District General Hospital, Oldham, U.K.*

Summary. Granulosa cells were aspirated 3–4 h before the expected time of ovulation from 10 follicles of 4 patients treated with gonadotrophins: 4 of the follicles were immediately preovulatory. The granulosa cells were cultured for 10 h with 17 α -hydroxypregnenolone or dehydroepiandrosterone and samples of medium removed at 3 and 10 h were assayed for 6 steroids.

Granulosa cells were unable to synthesize androgens from endogenous substrate or undertake conversions via the Δ^5 pathway, but cells from all follicles were capable of aromatizing exogenous androgens to oestrogens although this capability was reduced in cells from follicles beginning to luteinize. Granulosa cells from preovulatory follicles synthesized more progesterone from endogenous substrate than cells from follicles which had not begun to luteinize. The results provide further support for the two-cell theory of oestrogen biosynthesis whereby granulosa cells aromatize androgens which are synthesized by the thecal cells *in vivo*.

Introduction

Little is known about the cellular site of steroid synthesis in the human Graafian follicle. Thecal cells are thought to synthesize androgens and oestrogens from endogenous precursors and granulosa cells are known to produce progesterone after luteinization (Ryan & Petro, 1966; Channing, 1969; YoungLai, Edwards & Steptoe, 1972). Granulosa cells aspirated from human Graafian follicles 3–4 h before the expected time of ovulation and cultured *in vitro* convert pregnenolone to progesterone and 17 α -hydroxyprogesterone, and aromatize androgen substrates to oestrogen (Fowler, Fox, Edwards, Walters & Steptoe, 1978a). However, little or no oestradiol-17 β is produced if the only exogenous substrate is pregnenolone, suggesting that pregnenolone is not converted to androgens. The present study was to assess the utilization of Δ^5 steroids by human granulosa cells cultured in medium containing 17 α -hydroxypregnenolone (17 Δ^5 P) or dehydroepiandrosterone (DHA). Granulosa cells were taken from preovulatory follicles stimulated by human chorionic gonadotrophin as in the earlier study, and also from large follicles which had not begun to luteinize.

Materials and Methods

Patients

Granulosa cells were aspirated from the follicles of 4 patients treated with gonadotrophins (see Fowler *et al.*, 1978a). The patients were infertile because of tubal occlusion and were undergoing treatment by the removal of oocytes and the reimplantation of embryos (Edwards & Steptoe, 1975). All the patients were aware of the methods and treatments used and had given their informed consent. As shown in Text-fig. 1, Patients 2, 3 and 4 were given successive injections (i.m.) of human menopausal gonadotrophin (hMG: Pergonal, G. D. Searle & Co.) from Day 3 of the menstrual cycle, followed by a single injection of 5000 i.u. human chorionic gonadotrophin (hCG: Pregnyl, Organon Ltd) on Days 11 or 12, while Patient 1 was treated with hMG, and human luteinizing hormone (MRC LH). In order to assess the response of the patients to the gonadotrophins, 24-h urine samples were

collected for several days during and after the treatment. Total oestrogens and pregnanediol were measured by the methods described by Brown, Macleod, MacNaughton, Smith & Smyth (1968) and Rogers & Chamberlain (1972), respectively. Urinary LH was assayed by the method of Siddiqui, Jackson & Craig (1975).

Aspiration of follicles

Follicles were aspirated 32–33 h after the injection of hCG or LH, i.e. 3–4 h before the expected time of ovulation (Edwards & Steptoe, 1975). Single cells and large sheets of granulosa cells were obtained from 6 follicles of Patient 1 and from 4 follicles of Patients 2, 3 and 4. In all the patients, large follicles but no corpora lutea were observed. Six of the 10 Graafian follicles of Patient 1 were aspirated successfully and the appearance of large numbers of small clumps of granulosa cells indicated that the follicles were not immediately preovulatory (Fowler, Edwards, Walters, Chan & Steptoe, 1978b). Between 6 and 12 Graafian follicles were observed in Patients 2, 3 and 4 but only 4 follicles were used in this study (see Table 1). These follicles were preovulatory with large sheets of cells and viscous follicular fluid (Fowler *et al.*, 1978b). The cells were separated from the follicular fluid by centrifugation and the latter was stored at -20°C until assay. If sufficient was available the follicular fluid was assayed for progesterone and oestradiol-17 β so that the stage of development of the follicle could be assessed.

Culture of granulosa cells

The culture medium was Earle's balanced salt solution with the addition of 0.1 mg sodium pyruvate/100 ml, 0.75 g bovine serum albumin/100 ml and 100 i.u. penicillin/ml. Granulosa cells separated from follicular fluid were washed by suspension in culture medium and then centrifuged again. The endogenous concentrations of progesterone and oestradiol-17 β in 3 samples of granulosa cells after washing did not exceed 8 and 5 ng respectively.

The culture procedure followed was exactly that described by Fowler *et al.* (1978a). The cells were divided into two or three equal groups, as judged by microscopic examination of the size of the cell sheets in the aspirate, and placed in small tubes containing culture medium alone (controls) or medium with exogenous steroid substrate. All tubes were gassed with 5% CO₂, 5% O₂, and 90% N₂ and maintained at 37°C during the incubation period. The sheets of granulosa cells could not easily be dispersed, and cell counts were therefore made at the end of the culture period from squash preparations stained with methyl green. In the control tubes, the cells were suspended in 2.0 ml medium and used to measure the production of steroids from endogenous substrate: 0.5 ml was removed after 10 min to assess the concentration of steroids carried over from the follicular fluid at the beginning of culture. The cells in the other tubes were incubated with 1.5 ml culture medium containing 17 α -hydroxypregnenolone or DHA. The concentrations of exogenous substrate added to granulosa cell cultures (65–200 ng 17 α -hydroxypregnenolone and 800–1250 ng DHA: see Table 1) were adjusted to be close to the range found in follicular fluid (Fowler, Chan, Edwards, Walters & Steptoe, 1977). Samples of medium (0.5 ml) were removed from all cultures after incubation for 3 and 10 h. The production of various steroids was determined by subtracting the 10-min control value.

Radioimmunoassay of steroids

The radioimmunoassay procedures used were those described by Abraham, Hopper, Tulchinsky, Swerdloff & Odell (1971), Abraham, Buster, Kyle, Corrales & Teller (1973), Abraham, Manlimos, Solis, Garza & Maroulis (1975a) and Abraham, Manlimos, Solis & Wickman (1975b). Six steroids (progesterone, 17 α -hydroxyprogesterone, oestradiol-17 β , androstenedione, DHA and 17 α -hydroxypregnenolone) were assayed in all samples of culture medium. Testosterone was assayed in medium from Follicles 1–6. Only progesterone and oestradiol-17 β were assayed in follicular fluids. The specificities of the antisera, kindly supplied by Dr G. E. Abraham, were as described by Abraham *et al.* (1971, 1973, 1975a, b).

After extraction of the samples with ether, the steroids were separated on Celite microcolumns by using a stationary phase of ethylene glycol (Abraham, Buster, Lucas, Corrales & Teller, 1972).

Triplicate measurements were used to establish the standard curves for each assay and the steroid concentrations were estimated in two samples of each follicular fluid and culture medium sample. The concentration of steroids in the sample was calculated by interpolation from the standard curve by using a non-linear asymptotic model which had been fitted to the curve by iterative methods (Walters, 1974). The mean of the 3 blank samples was subtracted from the steroid levels before correction for recovery, the blank values being consistently low (<10 pg) in all assays. The measured values of known quantities of steroid added to the culture medium did not differ by more than 15% from the expected value. The smallest amount of steroid that could be measured per sample was 0.04 ng for progesterone, 17 α -hydroxyprogesterone, androstenedione and testosterone, 0.03 ng for oestradiol-17 β , and 0.125 ng for DHA and 17 α -hydroxypregnenolone. The precision of the assays was evaluated by duplicate measurements of the sample in the same or different assays. The average coefficient of variation ranged from 10.2 to 11.8% within assays and from 11.4 to 16.8% between assays.

Results

Urinary hormones

All 4 patients responded to treatment with hMG; urinary oestrogen values increased to ≥ 200 $\mu\text{g}/24$ h (Text-fig. 1). Pregnanediol values remained low in Patient 1, suggesting that luteinization had not occurred, but the rising levels of urinary pregnanediol (>6 mg/24 h) in Patients 2, 3 and 4 indicated that follicular luteinization had occurred in these women. There was no evidence of an endogenous LH surge before the hCG or LH injection except in Patient 2 in whom there was a rise in urinary LH on the day of hCG injection.

Text-fig. 1. Levels of urinary oestrogens and pregnanediol in the four patients treated with hMG and LH or hCG. L = time of laparoscopy.

Table 1. The production of steroids by granulosa cells, from women treated with gonadotrophins, cultured in the presence of 17 α -hydroxypregnenolone (17 Δ^5 P) or dehydroepiandrosterone (DHA)

Patient	Follicle	Steroids in follicular fluid (ng/ml)		Approximate no. of cells in culture	Precursor (ng)	Duration of culture (h)	Steroid production (ng/culture) [†]			
		Progesterone	Oestradiol-17 β				Progesterone	17 α -Hydroxy- progesterone	Androstenedione	Oestradiol-17 β
1	1	1092	2566	—*	None	10	10.45	15.41	<1.00	4.58
					DHA (1250)	3	2.62	9.72	5.37	67.46
					DHA (1250)	10	13.47	21.45	9.86	221.41
					17 Δ^5 P (65)	3	1.18	5.97	1.39	3.13
1	2	1881	3358	30 000	17 Δ^5 P (65)	10	6.20	14.73	<1.00	4.87
					None	3	5.59	4.81	<1.00	3.22
					None	10	10.78	11.33	<1.00	3.51
					DHA (1250)	3	12.28	6.79	5.18	67.30
1	3	1460	3060	325 000	17 Δ^5 P (65)	3	7.58	13.49	<1.00	3.59
					17 Δ^5 P (65)	10	9.73	19.75	<1.00	6.83
					None	3	37.32	35.82	<1.00	22.83
					None	10	81.55	86.23	<1.00	45.18
1	4	—	—	17 000	DHA (1250)	3	58.73	79.32	5.10	322.16
					DHA (1250)	10	144.79	131.26	7.80	508.22
					None	3	1.44	4.68	<1.00	4.64
					None	10	2.71	7.65	<1.00	6.66
					DHA (1250)	3	6.95	<1.00	2.19	103.95
					DHA (1250)	10	14.39	12.83	6.91	168.25
					17 Δ^5 P (65)	3	5.49	<1.00	<1.00	11.94
					17 Δ^5 P (65)	10	—	9.64	<1.00	12.51

1	5	1415	3033	50 000	None	3	1-58	3-49	<1-00	2-43
					None	10	18-50	26-16	<1-00	7-31
					17Δ ⁵ P (65)	3	3-37	14-13	<1-00	2-72
					17Δ ⁵ P (65)	10	26-29	55-49	<1-00	6-89
1	6	1073	3436	100 000	None	3	11-24	—	<1-00	7-08
					None	10	89-32	—	<1-00	13-36
					DHA (1250)	3	18-36	—	4-01	88-40
					DHA (1250)	10	65-83	—	20-89	346-00
					17Δ ⁵ P (65)	3	5-02	—	<1-00	5-48
					17Δ ⁵ P (65)	10	27-98	—	<1-00	6-63
2	7	14 334	1037	50 000	None	3	18-77	<1-00	<1-00	3-03
					None	10	59-17	1-80	<1-00	<1-00
					17Δ ⁵ P (200)	3	69-50	<1-00	<1-00	<1-00
					17Δ ⁵ P (200)	10	101-09	6-04	<1-00	<1-00
2	8	9205	218	—*	None	3	38-42	<1-00	<1-00	2-77
					None	10	208-64	17-79	<1-00	<1-00
					17Δ ⁵ P (200)	3	33-25	6-19	<1-00	<1-00
					17Δ ⁵ P (200)	10	156-36	26-41	<1-00	2-31
3	9	16 243	2929	600 000	None	10	718-59	58-46	2-15	22-83
					DHA (800)	10	1245-25	172-95	347-20	534-24
4	10	14 816	2418	50 000	None	3	95-16	—	<1-00	<1-00
					None	10	108-50	21-22	<1-00	<1-00
					DHA (1000)	3	141-47	—	123-15	68-94
					DHA (1000)	10	129-32	16-24	223-11	76-19

* Cells used for electron microscopy.
† The production of DHA and 17α-hydroxypregnenolone was < 1 ng in all cultures except those to which steroids were added as precursors.

Steroids in follicular fluid

The concentration of progesterone in the fluid of follicles from Patients 2, 3 and 4 (Table 1) was very high, confirming that the follicles were preovulatory and that luteinization had evidently begun. The concentration of progesterone was much lower in the follicular fluid taken from Patient 1, again confirming that luteinization had not been induced. The concentration of oestradiol-17 β in the follicular fluid of all but one of the follicles aspirated was high (>1000 ng/ml).

Steroid production by granulosa cells

Endogenous substrate. Very little progesterone was found in the control cultures of cells from Follicles 1–6 and this was especially evident when progesterone output was related to cell number (Tables 1 and 2). More progesterone was found in the cultures of granulosa cells from preovulatory follicles. Some 17 α -hydroxyprogesterone was produced from endogenous substrate. In general, only small amounts of oestradiol-17 β were present except for Follicles 3 and 9, and the output per cell was lower from Follicles 8–10 than from Follicles 1–6.

Table 2. Mean (range in parentheses) cellular output (calculated from data in Table 1) of progesterone, oestradiol-17 β and androstenedione by human granulosa cells cultured with dehydroepiandrosterone (DHA) or 17 α -hydroxypregnenolone (17 Δ^5 P)

Follicles	Treatment of patient	Precursor	Output of steroid in culture medium (ng/10 ³ cells/10 h incubation)		
			Progesterone	Oestradiol-17 β	Androstenedione
1–6	hMG + LH	None	*0.40 (0.16–0.89)	†0.18 (0.11–0.39)	0.0
		DHA	0.65 (0.45–0.85)	†4.26 (1.56–9.90)	0.20 (0.02–0.41)
		17 Δ^5 P	0.37 (0.27–0.53)	0.30 (0.13–0.72)	0.0
8–10	hMG + hCG	None	*1.29 (0.63–2.17)	0.01 (0.0–0.04)	0.0
		DHA	1.76 (1.44–2.08)	1.28 (0.89–1.66)	2.52 (0.58–4.46)
		17 Δ^5 P	2.02	0.0	0.0

* Mean difference ($\pm$ s.e.m.) 0.89 $\pm$ 0.34 (P < 0.05).

† Mean difference ($\pm$ s.e.m.) 4.08 $\pm$ 1.66 (P < 0.05).

Exogenous DHA. Substantial amounts of oestradiol-17 β were produced in all incubations (Table 1), the output per cell being highest in cultures of cells from follicles that had not begun to luteinize (Follicles 1–6). Only small amounts of androstenedione and no testosterone were detected. The rate of conversion of DHA to androstenedione was presumably the factor that limited oestrogen production in these cultures. In contrast, androstenedione accumulated in cultures of cells from Follicles 9 and 10, probably because of incomplete aromatization, and this was reflected in the lower output of oestradiol-17 β per cell (Table 2). Substantial quantities of DHA were utilized in all cultures (Table 3). It was not possible in this study to determine whether the addition of DHA to the culture medium affected progesterone output (see Hillier, Knazek & Ross, 1977) because the availability of precursor would have been a limiting factor.

Exogenous 17 α -hydroxypregnenolone. The production of progesterone and oestradiol-17 β remained similar to that in control incubations (Tables 1 and 2). No androgens were produced. Little or no 17 α -hydroxypregnenolone was utilized during the incubation, although small amounts may have been converted to 17 α -hydroxyprogesterone (Table 3). Presumably, most 17 α -hydroxyprogesterone is formed from progesterone.

Table 3. Utilization of dehydroepiandrosterone (DHA) and 17α -hydroxypregnenolone ($17\Delta^5$ P) and the production of androstenedione and oestradiol- 17β by human granulosa cells aspirated from follicles 3–4 h before ovulation and cultured for 10 h

Follicle	Substrate	Utilization of steroid* (ng)		Production of steroid† (ng)		
		Amount added	Amount utilized (%)	Androstenedione	Oestradiol	17-Hydroxyprogesterone
1	$17\Delta^5$ P	65	11 (17)	2	5	15
2		65	19 (29)	<1	5	20
4		65	2 (3)	<1	13	10
5		65	2 (3)	<1	7	56
6		65	11 (17)	<1	7	—
7		200	0 (0)	<1	1	6
8		200	0 (0)	<1	2	26
1	DHA	1250	266 (21)	11	221	
3		1250	364 (29)	8	508	
4		1250	240 (19)	7	168	
6		1250	660 (53)	21	346	
9		800	786 (98)	347	534	
10		1000	364 (36)	223	76	

* Obtained by subtracting the amount of steroid present in the culture medium at the beginning from that present at the end of the incubation.

† Obtained from data in Table 1.

Discussion

The type and pattern of steroid synthesis by granulosa cells *in vitro* will be conditioned by events in the follicle before aspiration. The increasing number of LH binding sites in growing follicles is associated with an increase in the ability of LH to stimulate adenylate cyclase activity and the production of cAMP (Ryan & Lee, 1976; Channing & Tsafiriri, 1977). Previous exposure of the follicle to FSH and to LH is a prerequisite for follicular growth and surge levels of LH may be necessary for luteinization because monkey granulosa cells fail to luteinize *in vitro* if removed before the LH surge *in vivo* (Channing, 1970). In addition, the LH surge reduces aromatizing activity in the follicles of sheep (Moor, 1974) and rabbits (LeMaire & Marsh, 1975) and the ovaries of rats (Katz & Armstrong, 1976).

Two distinct pathways of steroid biosynthesis exist in human granulosa cells exposed to gonadotrophins. Pregnenolone is converted to progesterone and 17α -hydroxyprogesterone via the Δ^4 pathway (Fowler *et al.*, 1978a) and androgens are aromatized to oestradiol- 17β . However, Δ^4 steroids are not converted to androgens, and pregnenolone and 17α -hydroxypregnenolone are not metabolized to DHA or other androgens via the Δ^5 pathway. The failure of granulosa cells to undertake these conversions may be due to the absence of the 17-hydroxylases and 17,20-lyases or to the inhibitory effects of steroids or other compounds in follicular fluid before the follicles were aspirated.

The two pathways of steroid synthesis by granulosa cells may be under independent control. Granulosa cells produce more progesterone from endogenous substrate as luteinization begins. LH may stimulate the production of pregnenolone from cholesterol (Hall & Young, 1968), thus increasing the availability of precursor, or increase the Δ^5 3 β -hydroxysteroid dehydrogenase/isomerase activity. The stimulation of the Δ^4 pathway would lead to increasing amounts of progesterone in follicular fluid as ovulation approaches. In contrast, the aromatizing activity of granulosa cells declines as luteinization proceeds and large quantities of androstenedione are not aromatized *in vitro*. A decrease in oestradiol synthesis has been observed in the rat after the injection of LH and was accompanied by a decline in the activity of the aromatizing enzymes and possibly a limitation of available substrate (Katz & Armstrong, 1976). DHA or other androgen precursors are presumably supplied by the thecal

cells for aromatization *in vivo*, as proposed in the two-cell theory of oestrogen synthesis (Falck, 1959) and supported by studies on hamsters and sheep (Makris & Ryan, 1975; Moor, 1977). The high concentrations of oestradiol-17 β in follicular fluid are probably due to the aromatizing activity of granulosa cells.

The production of progesterone and the aromatization of androgens to oestrogens may occur in the same or in different granulosa cells, or granulosa cells may switch from the production of one steroid to another in response to changes in their steroid and gonadotrophin receptors.

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