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REGULATION OF STEROID AND STEROID SULFATE PRODUCTION AND AROMATASE ACTIVITY IN CULTURED HUMAN GRANULOSA–LUTEAL CELLS

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Summary—The regulation of the production of steroids and steroid sulfates and the activity of aromatase in human luteinized granulosa cells were investigated. The cells were cultured for 48 h in the presence or absence of hCG and FSH. Basal production of pregnenolone (Pre, 0.3 ± 0.03 ng/μg protein) and progesterone (P, 19.3 ± 1.7 ng/μg protein) were high compared with that of other steroids beyond P in the steroidogenic pathway. The concentration of 17α-hydroxyprogesterone (17-OHP) was lower 0.17 ± 0.06 ng/μg and that of other steroids in the 4-ene and 5-ene pathways and steroid sulfates <0.05 ng/μg. Both hCG and FSH (100 ng/ml) stimulated the production of Pre and P 3- to 5-fold, but only minimal stimulation of other steroids and steroid sulfates was observed. Aromatase activity of granulosa–luteal cells was measured from the rate of formation of ^3H_2O from 1β-[^{3}H]androstenedione (1β[^{3}H]A) after exposing the cells to hCG, FSH or estradiol (E_2) for 48 h. Basal aromatase activity was relatively low, but hCG and FSH stimulated aromatase 8- and 4-fold, respectively. The incubation of granulosa–luteal cells with E_2 did not affect basal aromatase activity, but E_2 augmented FSH-stimulated aromatase 1.4-fold ($P < 0.025$). The results suggest that there is low 17α-hydroxylase and steroid sulfokinase activity in human granulosa–luteal cells. Aromatase activity in these cells is regulated by both hCG and FSH, and intra-ovarian estrogens may regulate granulosa cell aromatase activity.

INTRODUCTION

According to the two-cell theory, ovarian estrogen biosynthesis requires the interaction of both granulosa and theca cells [1–3]. Estrogens are formed through aromatization of the androgen precursors, androstenedione (A) and testosterone (T). Based primarily on studies in rats, it appears that the primary role of LH is to stimulate the biosynthesis of androgens from cholesterol in theca cells [4–7] while FSH acts on granulosa cells to stimulate aromatization. LH also has been shown to stimulate estrogen production directly [8, 9]. In 1968, Ryan *et al.* [10, 11] demonstrated that both granulosa and theca cells were needed for estrogen biosynthesis in human ovaries *in vitro*. It has been suggested in both animal and human studies that the inability of granulosa cells to produce androgens is due to the low activity of 17α-hydroxylase and/or 17,20-lyase enzymes [1, 7, 12–15], although 17α-hydroxylase activity also has been found in human granulosa cells [10, 11, 16, 17]. We have previously measured *P*450c17 mRNA accumulation by hybridization in human granulosa–luteal cells. Very small amounts of the message for this enzyme were detected reflecting low activity of both 17α-hydroxylase and 17,20-lyase [18].

The aim of this study was to examine the steroidogenic pathways of estrogen biosynthesis in human granulosa–luteal cells in greater detail. The capacity of human granulosa cells to produce steroid sulfates is not known. Therefore, in addition to the key steroids in the 4-ene and 5-ene pathways, we have measured the production of steroid sulfates. Additionally, the regulation of aromatase activity by gonadotropins and estradiol (E_2) in these cells was also investigated.

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EXPERIMENTAL

Granulosa cell culture

Human granulosa cells were obtained by follicular aspiration from women participating in the *in vitro* fertilization program. Follicular development was stimulated by treatment with clomiphene citrate (50 mg daily) on cycle days 3–7 and with human menopausal gonadotropin (hMG) 150 IU/day starting on cycle day 5 and thereafter for 4–5 days. The women received 5000 IU of hCG 36 h before follicular aspiration. Granulosa cells aspirated from follicles at least 15 mm dia were used in this study. After removing the ova, the remaining cells from each patient were pooled, washed twice with medium 199 (Gibco, Grand Island, NY, U.S.A.) and dispersed at 37°C for 30 min with gentle shaking in culture medium containing 0.1% collagenase–disperase (Boehringer–Mannheim, Mannheim, Germany). The dispersed cells were resuspended in 3 ml culture medium and layered onto 3.5 ml Ficoll–Paque (Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A.). Granulosa cells and red blood cells were separated by centrifugation at 600 *g* for 5 min. Granulosa cells accumulated at the interface between the culture medium and Ficoll–Paque, and red blood cells sedimented to the bottom of the tube. Granulosa cells were resuspended in medium 199 in Earle's balanced salt solution containing 10% fetal bovine serum (FBS), 2 mM glutamine and 50 μg/ml gentamicin. The cells were plated at a density of 2–4 $\times$ 10^5 cells/well (24 multiwells, Falcon) and cultured at 37°C in 95% air–5% CO_2. The media were changed every other day. After 6 days of culture, the cells were incubated with or without hCG (1, 10 and 100 ng/ml) or FSH (1, 10 and 100 ng/ml) for 48 h. After the incubations, the medium was collected for steroid and steroid sulfate measurements and stored at −20°C. Each experiment was performed with cells from individual patients.

Measurements of steroids and steroid sulfates

Unconjugated steroids were first extracted from cell culture media with diethylether–ethyl acetate 9:1, by vol). The organic phase was fractionated on Lipidex-5000™ microcolumns (Packar–Becker, B.V., Chemical Operations, Groningen, The Netherlands) [19, 20] followed by RIA of each steroid from the appropriate fraction using antisera of defined specificity [21]. E_2 was extracted from culture medium with diethylether–ethyl acetate and measured using an RIA kit provided by Farmos diagnostica (Oulunsalo, Finland).

Steroid sulfates were analyzed in the water phase of the mentioned extractions above [22]. The sulfate groups were cleaved by solvolysis in acidified ethyl acetate. The steroids were purified and fractionated on Lipidex-5000 microcolumns and assayed as described for unconjugated steroids. The intra- and interassay coefficients of variation were $<15\%$ for all steroids measured.

Aromatase assay

Aromatase activity was determined by the specific release of tritiated H_2O from 1β-[^{3}H]A. 1β-2β-[^{3}H]A (48.5 Ci/mmol) was purchased from New England Nuclear (Boston, MA, U.S.A.). 1β-[^{3}H]A was prepared by alkali reflux [23] and was purified on a celite column. Specifically, 4.0×10^5 granulosa–luteal cells were cultured for 6 days, after which the cells were exposed to hCG, FSH or E_2 for 48 h. Thereafter, the cells in culture plates were washed twice with medium 199 and incubated at 37°C (95% air–5% CO_2) for 3 h in medium 199 containing an excess of unlabeled A and 1β-[^{3}H]A, equivalent to a final concentration of 1 μM A. The reaction was stopped by transferring the media into glass vials containing 2 ml $CHCl_3$. The vials were centrifuged at 3000 rpm for 5 min and the upper aqueous layer pipetted into 12 $\times$ 75 mm glass test tubes with 30 mg of charcoal (activated, 20–20 mesh, EM Science, Granberry, NJ, U.S.A.). After vortexing, the tubes were centrifuged at 3000 rpm for 5 min. A 200 μl aliquot of the supernatant was pipetted into scintillation minivials with 4 ml Scintiverse II (Fisher Scientific, Santa Clara, CA, U.S.A.), vortexed, and the radioactivity measured. Nonspecific production of ^3H_2O was assessed by incubating 1β-[^{3}H]A in the absence of cells. The aromatization rate was expressed as fmol ^3H_2O/μg protein formed during the 3 h incubation. Aromatase activity increased linearly up to 180 min, and incubation with 0–5 μM concentrations of A gave a K_m of 0.067 μM (Fig. 1). The inhibition of aromatase activity was tested using a potent aromatase inhibitor, 4-hydroxyandrostenedione (Fig. 2).

Protein determination

The culture wells were washed twice with PBS, and the cells removed with 0.5 N NaOH.

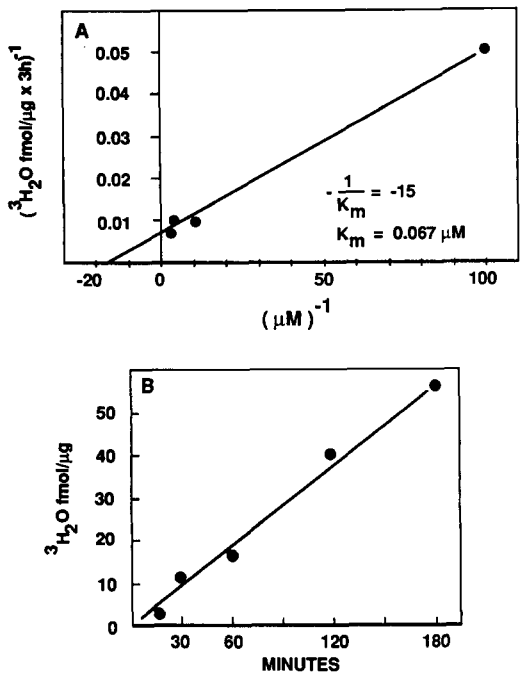

Fig. 1. Aromatase assay. (A) 4×10^5 Granulosa–luteal cells were incubated for 3 h with varying A concentrations (0–5 μM). (B) 4×10^5 cells were incubated for 0–180 min with 1 μM A. Each point represents the mean ± SD of 3 measurements.

Protein concentration was determined by the method of Bradford [24].

Other materials

hCG (hCG CR-121) was provided by the Center for Population Research of the NICHHD (NIH, Bethesda, MD, U.S.A.). The biological potency of hCG was 13,450 IU/mg. FSH (hFSH-3) was provided by the NIADDK through the National Hormone and Pituitary Program. hFSH-3 contained 3100 IU FSH/mg and 400 IU LH/mg by bioassay. Estradiol-17β was obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.).

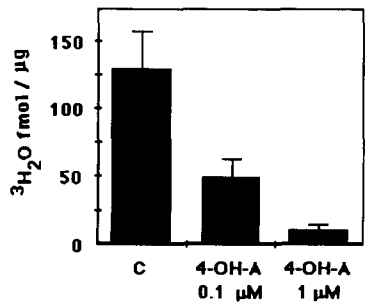

Fig. 2. Effect of 4-hydroxyandostenedione (4-OH-A) on granulosa–luteal cell aromatase activity during 3 h incubation. Each value represents the mean ± SD of 3 determinations.

Data analysis

Statistical comparisons were made by one-way analysis of variance (ANOVA). Comparable results were obtained in at least 3 separate experiments in the cells from different women.

RESULTS

Basal production of pregnenolone (Pre) and progesterone (P) during 48 h incubation was 0.32 ± 0.03 and 19.3 ± 1.7 ng/μg protein, respectively, which was clearly higher than the production of the other steroids beyond P in the steroidogenic pathway. The basal production of 17α-hydroxyprogesterone (17-OHP) was 0.17 ± 0.06 ng/μg protein and the concentrations of other steroids measured were < 0.04 ng/μg protein (Figs 3 and 4). Both hCG and FSH stimulated the production of Pre and P, but only minor stimulation ($P > 0.05$) was observed in the production of other steroids. The stimulated concentrations of Pre and P after the 100 ng/ml dose of hCG were 1.7 ± 0.2

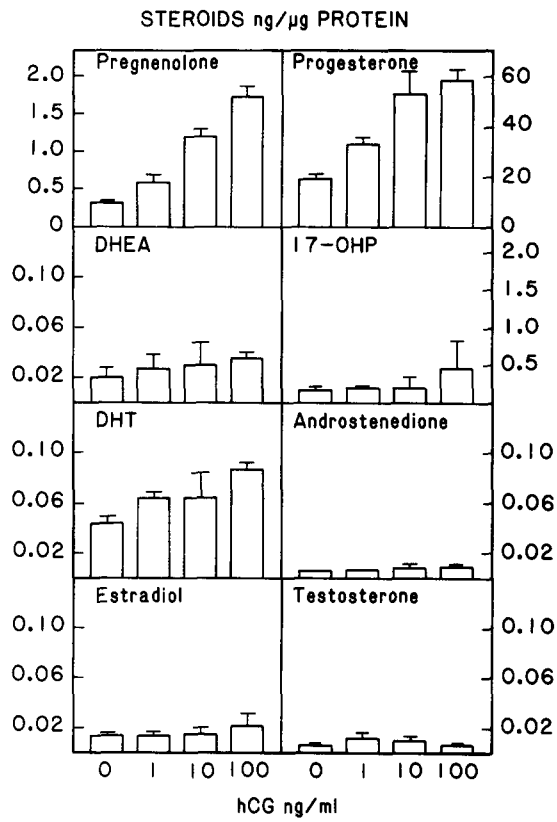

Fig. 3. Effect of different doses of hCG on steroid production in human granulosa–luteal cells during 48 h incubation. The cells were first cultured for 6 days in plain culture medium, whereafter they were exposed to hCG or FSH for 48 h. The results represent the mean ± SD of 3 determinations.

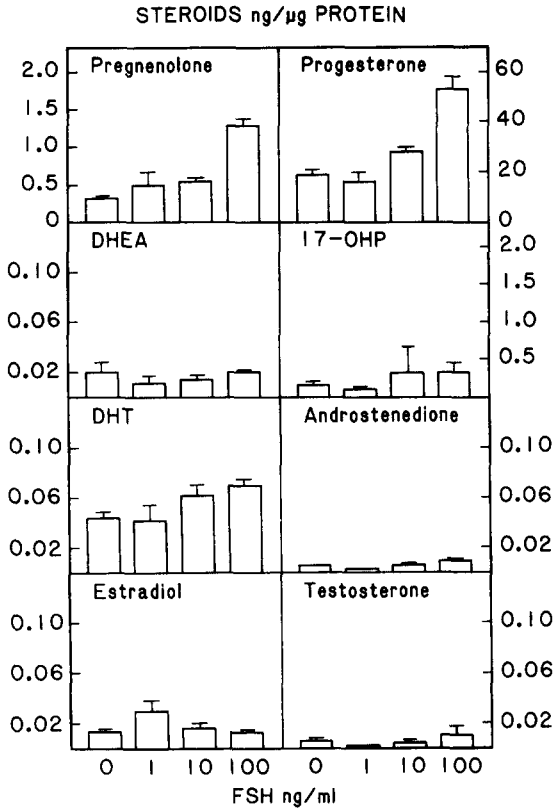

Fig. 4. Effect of different doses of FSH on steroid production in human granulosa–luteal cells during 48 h incubation. The cells were first cultured for 6 days in plain culture medium, whereafter they were exposed to hCG or FSH for 48 h. The results represent the mean ± SD of 3 determinations.

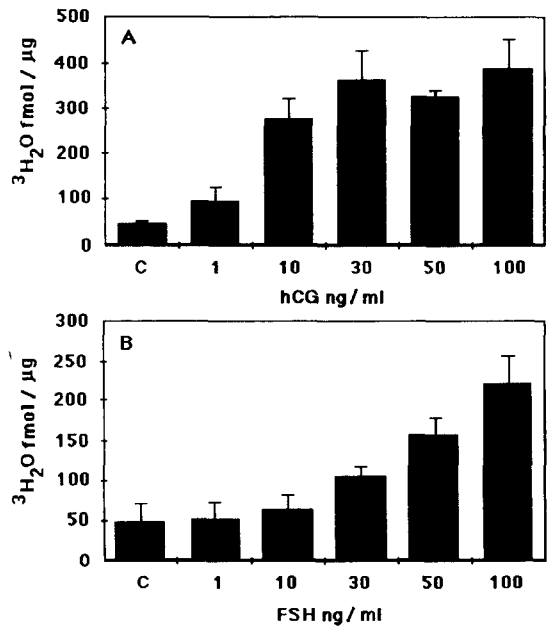

Fig. 5. Effect of different doses of hCG (A) and FSH (B) on aromatase activity in human granulosa–luteal cells during 48 h incubation. The cells were first cultured for 6 days in plain culture medium, whereafter they were exposed to hCG or FSH for 48 h. The results represent the mean ± SD of 3 determinations.

and 59.3 ± 4.2 ng/μg protein, respectively ($P < 0.001$), and those after 100 ng/ml FSH 1.3 ± 0.2 ($P < 0.001$) and 53.3 ± 6.4 ng/μg protein ($P < 0.001$), respectively.

Basal and gonadotropin-stimulated concentrations of the steroid sulfates were low. The highest gonadotropin-stimulated concentration was found for 5-androstene-3β,17β-diol 3-sulfate, 0.2 ng/μg protein. The basal concentrations of all other steroid sulfates measured were <0.01 ng/μg and the gonadotropin-stimulated concentrations <0.1 ng/μg (Table 1), although stimulation of Pre sulfate by both hCG and FSH was observed at the 100 ng/ml concentrations of both trophic hormones.

The basal aromatase activity of cultured granulosa–luteal cells was low. After exposing the cells to 100 ng/ml of hCG or FSH, the aromatase activity increased from 43.9 ± 9.4 to 385.6 ± 66.2 fmol/μg protein and from 49.5 ± 18.7 to 221.2 ± 35.3 fmol/μg protein, respectively (Fig. 5). The incubation of granulosa–luteal cells with E_2 ($10^{-10} - 10^{-6}$ M) did not affect basal aromatase activity, but concomitant incubation with FSH (30 ng/ml) and E_2 increased FSH-induced aromatase activity from 176.6 ± 34.2 to 250.8 ± 37.2 fmol/μg protein ($P < 0.05$) (Fig. 6).

DISCUSSION

These results show high and cumulative production of P in cultured human granulosa cells, reflecting luteinization of at least a portion of the cells. The cells were obtained from preovulatory follicles induced by exogenous

Table 1. Production of steroid sulfates by cultured human granulosa–luteal cells in response to hCG and FSH during 48 h incubation, mean ± SD

	hCG (ng/ml)				FSH (ng/ml)		
Protein (pg/μg)	0	1	10	100	1	10	100
Pregnenolone sulfate	9.2 ± 3.4	9.4 ± 12.0	8.0 ± 7.9	18.5 ± 1.7	23.9 ± 8.9	72.4 ± 35.4	58.6 ± 18.6
17α-OHP sulfate	1.1 ± 1.0	2.5 ± 4.3	41. ± 3.6	1.2 ± 1.6	3.5 ± 3.7	19.0 ± 5.0	2.4 ± 3.1
DHEAS	10.6 ± 2.3	10.5 ± 1.7	8.2 ± 3.6	8.8 ± 1.3	14.4 ± 4.9	30.0 ± 16.5	18.6 ± 2.4
5-Androstene-3β,17β-diol 3-sulfate	57.0 ± 3.5	84.2 ± 5.1	163.4 ± 110.6	110.2 ± 75.2	112.5 ± 18.7	204.2 ± 142.2	82.4 ± 29.6
Testosterone sulfate	1.1 ± 0.3	2.7 ± 2.5	1.6 ± 0.1	1.9 ± 0.8	2.6 ± 1.1	5.4 ± 1.2	5.6 ± 0.7

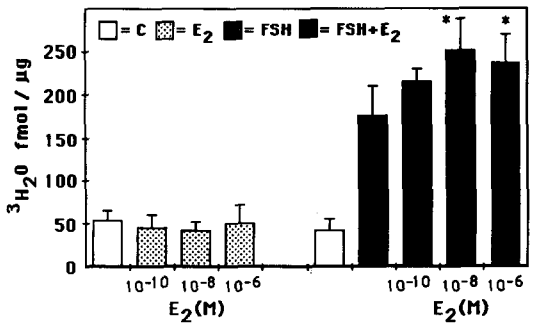

Fig. 6. Effect of E_2 on human granulosa–luteal cell aromatase activity. Granulosa–luteal cells were cultured for 48 h in the presence or absence of 30 ng/ml FSH with or without increasing concentrations of E_2. After the culture, the cells were washed with medium 199 and aromatase activity was determined by the release of ^3H_2O from 1β-[^{3}H]A during 3 h incubation. The results represent the mean ± SD of 3 determinations. *$P < 0.05$ vs FSH-stimulated level.

gonadotropins and clomiphene citrate, which may affect the function of these cells in the culture.

Both hCG and FSH stimulated P production in a dose-dependent manner. In lower concentrations (1 and 10 ng/ml) hCG seemed to be a more potent stimulator than FSH, but no difference was seen when the concentration of 100 ng/ml was used. This is consistent with our earlier studies, where 50 ng/ml and lower doses of hCG stimulated *P*450scc mRNA accumulation and P production more than the equivalent concentration of FSH [18, 25]. However, the effect of the contamination of FSH with LH can not be excluded. The production of P was cumulative and clearly higher than that of Pre, which reflects, on the other hand, high 3β-hydroxysteroid dehydrogenase activity and on the other hand a steroidogenic block distally to P production. The production of 17-OHP and other steroids beyond P were low supporting the classical two-cell theory that the conversion of P to androgens is low or absent in granulosa cells [1, 7, 26, 27]. Accordingly, very little *P*450c17 mRNA has been found in human granulosa–luteal cells [18], and exogenous androgen substrate is needed for estrogen synthesis in human granulosa cells *in vitro* [7, 12–15]. As other investigators have reported contrasting results [10, 11, 16, 17], it is possible that the activity of 17α-hydroxylase in granulosa cells depends, for example, on the stage of the follicular development.

In this study, we measured directly the catalytic activity of aromatase in human granulosa–luteal cells. Both hCG and FSH were able to stimulate aromatase in a dose-dependent manner. FSH has been considered to be the prime inducer of aromatase activity in rat and human granulosa cells [5, 13, 15, 28], but LH/hCG also has been shown to stimulate aromatase in human granulosa cells [29, 30] and in FSH-primed rat granulosa cells [8, 9]. In the present study, hCG increased aromatase activity to an even greater degree than FSH, which is consistent with a previous report of Hillier *et al.* [31] showing that granulosa cells collected during the early and mid-follicular phase produce E_2 in response to FSH, but the response is lost in cells from mature follicles.

Our results demonstrate that E_2 augments that FSH stimulation of aromatase activity in human granulosa–luteal cells. The direct regulation of aromatase activity by estrogens was first demonstrated in rats by Adashi and Hsueh [32]. They showed that the minimal effective dose of E_2 was 3.7×10^{-10} M, which is within the same range as the effective dose in human granulosa–luteal cells. Our findings also indicate that intrafollicular estrogens may stimulate aromatase activity directly in granulosa–luteal cells, increasing their own production rate.

The production of steroid sulfates in human granulosa–luteal cells has not been studied previously. The biological function of steroid sulfates in steroid-producing cells is obscure. However, in the human testis, steroid sulfates are produced in high amounts and it has been suggested that they serve as steroid reservoirs and precursors in the biosynthesis of unconjugated steroids [33, 34]. In this study, the accumulation of steroid sulfates in the culture medium was low, reflecting either a low degree of formation or rapid hydrolysis of these conjugates. Assuming that the same enzymes are functioning in the sulfate pathway as in the free 4-ene and 5-ene steroid pathways, the low formation of steroid sulfates could be due to the low activity of 17α-hydroxylase. However, since the production of Pre sulfate was also low, it is more likely that steroid sulfokinase is the rate limiting step in the formation of steroid sulfates in granulosa–luteal cells. Consonant with these *in vitro* studies, the concentrations of steroid sulfates are the same in human ovarian and peripheral venous blood, suggesting a low degree of formation of sulfated steroids in the ovary [35].

In conclusion, there is high and cumulative production of P in cultured human granulosa–luteal cells, but the production of steroids

beyond P in the steroidogenic pathway is limited, reflecting the low activity of 17α-hydroxylase. Aromatase activity is stimulated by hCG and FSH, and E_2 augments the stimulation of aromatase activity by FSH. These findings support the two-cell theory of ovarian estrogen biosynthesis. The production of steroid sulfates in cultured human granulosa–luteal cells is minimal, reflecting low steroid sulfokinase activity.

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