Estrogens Augment the Stimulation of Ovarian Aromatase Activity by Follicle-stimulating Hormone in Cultured Rat Granulosa Cells*

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Eli Y. Adashi§ and Aaron J. W. Hsueh§
From the Department of Reproductive Medicine, University of California, San Diego, La Jolla, California 92039

The effects of estrogens on ovarian aromatase activity were investigated in vitro using granulosa cells from immature hypophysectomized estrogen-primed rats. The cells were cultured for 3 days in an androgen-free medium in the presence of follicle-stimulating hormone (FSH), with or without the specified estrogen. After washing, the cells were reincubated for 5 h with 10^{-7} M androstenedione, and the formation of estrogens was measured. Estrogen production by control and diethylstilbestrol-treated cells was negligible, while FSH-stimulated aromatase activity was measured. The intermittent treatment with diethylstilbestrol led to dose-dependent increases in the FSH-induced aromatase activity with an ED_{50} value of 4 x 10^{-7} M and an apparent V_{max} value 12- to 16-fold higher than those induced by FSH alone. The direct stimulatory effect of estrogens was time-dependent and was not accounted for by increases in cell protein. Various native and synthetic estrogens also augmented the FSH induction of aromatases (native estrogens: estradiol-17β = estrone > estradiol-17α > estritol; synthetic estrogens: hexestrol > moxestrol > ethinyl estradiol >>> chlorotrianisene and mestranol). The effect of estradiol-17β was dose-dependent with an ED_{50} value of 9 x 10^{-8} M, which is within the physiological levels of follicular estradiol-17β. Although treatment with androgens also enhanced the FSH-induced aromatases, treatment with a progestin (R5020) or a mineralocorticoid (aldosterone) was without effect. Thus, estrogens directly augment the FSH stimulation of granulosa cell aromatase activity by FSH. Follicular estrogens may activate intraovarian aromatases, further increasing their activity and the preovulatory estrogen surge.

It is now well accepted that follicle-stimulating hormone is the prime inducer of aromatase activity in rat (1-6) and human (7) ovaries based on in vitro (1, 2, 4-7) and in vivo (3, 5) studies on granulosa cells. However, the possibility that this effect of FSH\(^1\) is subject to modulation by various steroid hormones has thus far received little attention. Although androgens have recently been shown to synergize with FSH in the induction of aromatase activity in rat granulosa cells (8), the possible involvement of estrogens has not been studied.

Estrogens have been implicated in several ways in the regulation of granulosa cell differentiation. Specifically, estrogen treatment of immature hypophysectomized female rats results in increased ovarian weight due to the proliferation of granulosa cells and growth of preantral follicles (9-23). Estrogens have also been reported to exert an antiatretic effect (13, 17, 18, 24), to increase granulosa cell estrogen receptor content (20), and to increase the coupling of granulosa cells through intercellular gap junctions (25). In addition, estrogens have been shown to synergize with FSH in the stimulation of follicular antrum formation (11, 16, 17, 19, 20, 22) and the induction of granulosa cell luteinizing hormone receptors (26, 27).

In view of the ability of estrogens to augment a broad range of FSH-dependent functions, it is of interest to study the interaction of estrogens with FSH in one of the key functions of ovarian follicles, i.e., the ability to synthesize estrogens through the activation of aromatases. Our present findings in cultured granulosa cells indicate that estrogens augment the FSH stimulation of aromatase activity thereby exerting a direct and positive autoregulatory feedback action on their own production.

**EXPERIMENTAL PROCEDURES**

**Animals—**Immature (23-25 days old) Sprague-Dawley female rats were hypophysectomized by Johnson Laboratories, Inc. (Bridgeview, IL) and delivered on the second postoperative day. Silastic capsules (10 mm) containing diethylstilbestrol were implanted subcutaneously at the time of hypophysectomy (5). The animals were housed in air-conditioned quarters and given physiological saline (0.9% NaCl) solution and a mixture of bread, milk, and tap water ad libitum. A 14:10-h light-dark cycle was maintained with the light cycle starting at 0600 h. The animals were sacrificed by cervical dislocation between 4-6 days after surgery. In some experiments, immature hypophysectomized Sprague-Dawley rats without diethylstilbestrol implants were also used between 4-6 days after surgery.

**Reagents and Hormones—**McCoy’s 5a medium (modified, without serum), penicillin-streptomycin solution, L-glutamine, and trypan blue stain (0.4%) were obtained from Grand Island Biological Co. (Grand Island, NY). Androstenedione (4-androstene-3,17-dione), diethylstilbestrol (3,4-bis-(p-hydroxyphenyl)3-hexene), dihydrotestosterone (5α-androstan-3α,17β-diol-3-one), aldosterone (4-pregnen-18α,11β,21-diol-3,20-dione), 17β-estradiol (E2-17β, 1,3,5(10)-estratrien-3,17β-diol), 17α-estradiol (E2-17α, 1,3,5(10)-estratrien-3,17β-diol), estrone (E1, 1,3,5(10)-estraatrien-3,17β-triol), estradiol (E2, 1,3,5(10)-estraatrien-3,17β-diol), 17α-estradiol (E2-17α, 1,3,5(10)-estratrien-3,17β-diol), ethinyl estradiol (1,3,5(10)-estraatrien-3α-ethyl-17β-diol), and mestranol (1,3,5(10)-estratrien-17α-ethyl-3,17β-diol-3-methylether) were from Sigma. Methyltrienolone-

\[\text{E}-17β, 17β-estradiol; E₁, estrone; E₂-17α, 17α-estradiol; E₂, estradiol; R1881, methyltrienolone.}\]
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The estrogen content of the medium was determined by radioimmunoassay of unextracted medium using an antisera (S-310#5) provided by Dr. G. Abraham (5). This antisera cross-reacts 100% with E2, E3, and E1, but shows no cross-reactivity. In the test interval. The presence or absence of diethylstilbestrol during the test interval was determined by radioimmunoassay of unextracted medium using an antisera (S-310#5) and with or without increasing concentrations (10^{-9}-10^{-7} M) of diethylstibestrol. Following this interval, the cells were washed twice and reincubated for a 5-h test interval in an androstenedione (10^{-7} M)-supplemented medium (Fig. 1). The estrogen production by control and diethylstibestrol (10^{-7} M)-treated cells was negligible, whereas treatment with FSH brought about a substantial increase in aromatase activity (estrogen accumulation: 7.5 ± 1.0 ng/mg of protein/5 h). Furthermore, concomitant treatment with increasing concentrations (10^{-10}-10^{-7} M) of diethylstibestrol led to dose-dependent increases in the FSH-induced aromatase activity with an ED_{50} value of 4.0 ± 0.5 × 10^{-8} M.

RESULTS

Effect of Treatment with Diethylstibestrol on Basal and FSH-induced Aromatase Activity in Rat Granulosa Cells—To evaluate the effect of treatment with diethylstibestrol (a nonsteroidal estrogen) on basal and FSH-induced aromatase activity, granulosa cells were cultured for 3 days in the presence or absence of FSH (10 ng/ml) with or without increasing concentrations (10^{-9}-10^{-7} M) of diethylstibestrol. Following this interval, the cells were washed twice and reincubated for a 5-h test interval in an androstenedione (10^{-7} M)-supplemented medium. The estrogen production by control and diethylstibestrol (10^{-7} M)-treated cells was negligible, whereas treatment with FSH brought about a substantial increase in aromatase activity (estrogen accumulation: 7.5 ± 1.0 ng/mg of protein/5 h). Furthermore, concomitant treatment with increasing concentrations (10^{-10}-10^{-7} M) of diethylstibestrol led to dose-dependent increases in the FSH-induced aromatase activity with an ED_{50} value of 4.0 ± 0.5 × 10^{-8} M.
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10^{-9} \text{ M}. The maximal activity stimulated by 10^{-7} \text{ M} diethylstilbestrol and FSH was 12-fold higher than that induced by FSH alone. These findings indicate that diethylstilbestrol augments the FSH stimulation of aromatase activity in a dose-dependent manner.

**Effect of Other Steroid Hormones on the FSH Induction of Aromatase Activity**—To examine the specificity of the stimulatory effect of diethylstilbestrol, granulosa cells were cultured for 3 days in the presence or absence of FSH (10 ng/ml) with or without 10^{-7} \text{ M} diethylstilbestrol, dihydrotestosterone (a nonaromatizable androgen), R1881 (a synthetic androgen), or aldosterone (a mineralocorticoid) (Fig. 2). Concomitant treatment with diethylstilbestrol, dihydrotestosterone, and R1881 brought about 14-, 9-, and 13-fold increases in the FSH-induced aromatase activity, respectively. In contrast, treatment with aldosterone was without effect. Furthermore, treatment with 10^{-7} \text{ M} of R5020 (a synthetic progestin) did not affect the FSH induction of aromatase activity (estrogen production (ng/mg of protein/5 h): FSH-treated = 8.4 ± 1.4; FSH plus R5020-treated, 10.3 ± 1.0). These findings indicate that the ability of diethylstilbestrol to augment the FSH induction of aromatase activity is shared by native and synthetic androgens but not by mineralocorticoids and progestins.

**Time Dependence of the Effect of Treatment with Diethylstilbestrol and FSH on Aromatase Activity**—To evaluate the time course of the effect of treatment with diethylstilbestrol and FSH on the aromatase activity, granulosa cells were cultured for increasing periods of time (1-3 days) in the presence or absence of FSH (10 ng/ml) with or without 10^{-7} \text{ M} diethylstilbestrol (Fig. 3). Treatment with FSH by itself led to progressive time-dependent increases in aromatase activity, becoming significant at 2 days after incubation. Although concomitant treatment with diethylstilbestrol for 1 day was without effect on the FSH-induced aromatase activity, treatment for 2 and 3 days led to 7- and 12-fold increases in the FSH effect, respectively. These findings indicate that the stimulatory effect of diethylstilbestrol and FSH is time-dependent and that it becomes operative within 2 days of treatment.

![Fig. 3. Time dependence of the effect of treatment with diethylstilbestrol on the FSH-induced aromatase activity. Granulosa cells were cultured as described in Fig. 1 for increasing periods of time (1-3 days) in the presence or absence of FSH (10 ng/ml) with or without 10^{-7} \text{ M} diethylstilbestrol. This was followed by treating the cells with 10^{-3} \text{ M} androstenedione for 5 h. Media estrogen was measured by radioimmunoassay. The results represent the mean ± S.E. of triplicate determinations. C, controls. DES, diethylstilbestrol.](image-url)

![Fig. 4. Effect of treatment with native estrogens on the FSH-induced aromatase activity. Granulosa cells were cultured as described in Fig. 1 in the presence or absence of FSH (10 ng/ml) with or without increasing concentrations (10^{-10}-10^{-7} \text{ M}) of E_2-17\beta, E_1, E_3, or E_2-17\alpha. Media estrogen was measured by radioimmunoassay. The results represent the mean ± S.E. of triplicate cultures. C, controls.](image-url)

**Effect of Treatment with Native Estrogens on the FSH Induction of Aromatase Activity**—To examine the effects of treatment with native estrogens on the FSH induction of aromatase activity, granulosa cells were cultured for 3 days in the presence or absence of FSH (10 ng/ml) with or without increasing concentrations (10^{-10}-10^{-7} \text{ M}) of E_2-17\beta, E_1, E_3, or E_2-17\alpha (Fig. 4). Concomitant treatment with increasing concentrations of E_2-17\beta or E_1 brought about identical dose-
Dependent increases in the FSH-induced aromatase activity with an ED50 value of 9.2 ± 0.7 × 10^{-9} M and a maximal activity 8-fold higher than that induced by FSH alone. The projected minimal effective dose (defined as the dose leading to an increase of 2 standard deviations over FSH-induced aromatase activity) was 3.7 ± 0.3 × 10^{-9} M for both E2-17β and E1. Although concomitant treatment with E1 and E2-17α also led to dose-dependent increases in the FSH-induced aromatase activity, the maximal levels of aromatase in cells treated with FSH and 10^{-7} M of the estrogens were only 3- to 4-fold higher than those achieved by FSH alone. Since the effects of E1 and E2-17α did not plateau at the highest dose used, these data did not permit accurate determination of ED50 values. However, estimations suggest that the ED50 values for these steroids are at least one order of magnitude higher than those for E2-17β and E1. These findings indicate that these native estrogens augment the FSH induction of aromatase activity (rank order of potency: E2-17β > E1 > E2-17α > E3).

Effect of Treatment with Synthetic Estrogens on the FSH Induction of Aromatase Activity—To evaluate the effect of treatment with synthetic estrogens on the FSH induction of aromatase activity, granulosa cells were cultured for 3 days in the presence or absence of FSH (10 ng/ml) with or without increasing concentrations (10^{-9} - 10^{-7} M) of various synthetic estrogens (Fig. 5, A and B). Concomitant treatment with increasing concentrations of hexestrol, moxestrol, or ethinyl estradiol led to dose-dependent increases in the FSH-induced aromatase activity with ED50 values of 2.0 ± 0.4 × 10^{-9}, 9.3 ± 0.6 × 10^{-9}, and 1.0 ± 0.4 × 10^{-8} M, respectively. However, concomitant treatments with increasing concentrations of chlorotrianisene or mestranol were virtually without effect. These findings indicate that these synthetic estrogens augment the FSH induction of aromatase activity (rank order of potency: hexestrol > moxestrol > ethinyl estradiol; 5.0:1.1:1.0).

Effect of Treatment with Diethylstilbestrol and FSH on the Apparent Kinetic Parameters of the Aromatase Enzymes—To determine the effect of treatment with diethylstilbestrol on the apparent Kₜₐ and Vₜ₉ₙ values of the FSH-induced aromatase enzymes, granulosa cells (3 × 10^5 viable cells/culture) were cultured for 3 days in the presence of FSH (10 ng/ml) with or without 10^{-7} M diethylstilbestrol. At the end of this incubation interval, the media were removed and the cells were washed and reincubated for a 5-h interval in media containing increasing concentrations (1-1000 nM) of androstenedione (Fig. 6, A and B). As shown in Fig. 6A, provision of increasing concentrations of androstenedione led to dose-dependent increases in the accumulation of estrogen in cells treated with FSH or FSH plus diethylstilbestrol. Under the saturating dose (1000 nM) of androstenedione, cells primed with both FSH and diethylstilbestrol accumulated about 16 times more estrogen as did cells treated with FSH alone. Lineweaver-Burk plot analysis of the data (Fig. 6B) revealed apparent Vₜ₉ₙ values of 4.9 and 79 ng/ng of protein/5 h for cells treated with FSH and FSH plus diethylstilbestrol, respectively. The corresponding apparent Kₜₐ values were 6.6 and 26.4 × 10^{-8} M. These findings indicate that concomitant treatment with diethylstilbestrol brings about a 16-fold increase in the maximal activity of the FSH-induced aromatase enzymes.

Effect of Treatment with Diethylstilbestrol on the FSH Induction of Aromatase Activity in Granulosa Cells from Hypophysectomized Rats without Diethylstilbestrol Implant—To evaluate the effect of diethylstilbestrol priming in vivo on the in vitro action of the estrogens, granulosa cells were obtained from immature hypophysectomized female rats not bearing diethylstilbestrol capsules and cultured for 3 days in the presence or absence of FSH (10 ng/ml) with or without increasing concentrations (10^{-10} - 10^{-7} M) of diethylstilbestrol (Fig. 7). Concomitant treatment with increasing concentrations of diethylstilbestrol led to dose-dependent increases in the FSH-induced aromatase activity with an ED₅₀ value of 7.5 × 10^{-10} M and a maximal activity 4-fold higher than that induced by FSH alone. These findings indicate that diethylstilbestrol augments the FSH stimulation of aromatase activity in granulosa cells not primed with the estrogen in vivo.
Augmentation of Aromatase Activity by Estrogens

FIG. 6. Effect of treatment with diethylstilbestrol on apparent kinetic parameters of the FSH-induced aromatases. Granulosa cells were cultured as described in Fig. 1 in the presence or absence of FSH (10 ng/ml) with or without $10^{-7}$ M diethylstilbestrol. At the end of this induction interval, the medium was removed and the cells were washed with medium and reincubated for a 5-h test interval with increasing concentrations (1-1000 nM) of androstenedione. Media estrogen was measured by radioimmunoassay. A, enzyme saturation plots; B, Lineweaver-Burk plot analysis. The results represent the mean ± S.E. of triplicate cultures. DES, diethylstilbestrol.

FIG. 7. Effect of in vitro treatment with diethylstilbestrol on the FSH-induced aromatase activity of granulosa cells from rats without diethylstilbestrol capsules. Granulosa cells were obtained from immature hypophysectomized female rats not bearing diethylstilbestrol capsules and cultured as described in Fig. 1 in the presence or absence of FSH (10 ng/ml) with or without $10^{-7}$ M diethylstilbestrol, E$_2$-17$\beta$, or E$_1$. Determinations were carried out as described under "Experimental Procedures." Data points represent the mean ± S.E. of triplicate determinations.

Effect of Treatment with FSH and Estrogens on the Cellular Protein Content—To determine the effects of treatment with FSH and estrogens on the cellular protein content, granulosa cells were cultured for 3 days in the presence or absence of FSH (10 ng/ml) with or without $10^{-7}$ M diethylstilbestrol, E$_2$-17$\beta$, or E$_1$. At the end of the incubation, the cells were washed with medium and reincubated for 5 additional hours, and the total cellular protein content was measured (Table I). Treatment with FSH led to a 30% increase in the protein content of granulosa cells as compared with controls. However, concomitant treatment with diethylstilbestrol, E$_2$-17$\beta$, or E$_1$ did not lead to significant alterations in the protein content as compared with cells treated with FSH alone.

DISCUSSION

The modulatory role of estrogens in the induction of ovarian aromatase activity by FSH was investigated in vitro using granulosa cells from immature hypophysectomized female rats. Our findings indicate that various native and synthetic estrogens augment the FSH stimulation of aromatase activity. This direct and specific effect of estrogens is associated with

TABLE I

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cellular protein (µg/culture)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>12.1 ± 0.4</td>
</tr>
<tr>
<td>Diethylstilbestrol</td>
<td>13.4 ± 1.0</td>
</tr>
<tr>
<td>E$_2$-17$\beta$</td>
<td>12.8 ± 0.7</td>
</tr>
<tr>
<td>E$_1$</td>
<td>11.5 ± 0.4</td>
</tr>
<tr>
<td>FSH</td>
<td>16.0 ± 0.5$^*$</td>
</tr>
<tr>
<td>FSH + diethylstilbestrol</td>
<td>15.8 ± 0.9$^*$</td>
</tr>
<tr>
<td>FSH + E$_2$-17$\beta$</td>
<td>14.7 ± 0.4$^*$</td>
</tr>
<tr>
<td>FSH + E$_1$</td>
<td>16.2 ± 0.4$^*$</td>
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</tbody>
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$^*$Significantly ($p < 0.05$) different from untreated cells.
an increase in the apparent maximal velocity of the enzymes and is time- and dose-dependent.

The present findings constitute the first in vitro evidence to demonstrate the ability of estrogens to augment the FSH stimulation of aromatase activity in rat granulosa cells. Although the role of FSH in the induction of aromatases has been amply documented (1-6), possible estrogenic modulation of this process could not be evaluated as previous studies were conducted with media supplemented with aromatizable androgens. Since androgens may convert to estrogens during the induction period and may enhance the accumulation of progesterins (32-35), use of androgen-supplemented media precludes studies of the interaction between FSH and the various sex steroids. In fact, formerly reported "FSH-induced" aromatase activity probably represents the sum effect of FSH and some of the medium steroids. Given these considerations, this study employed an androgen-free induction period to investigate the effects of estrogens on the FSH-induced aromatase activity. Similar reasoning has recently prompted the use of an androgen-free induction period to study the interaction between androgens and FSH on aromatase activity (8, 36).

Our findings indicate that concomitant treatment with 10^{-7} M diethylstilbestrol results in a 12- to 16-fold increase in the apparent maximal velocity (V_{max}) of the FSH-induced aromatases along with a 4-fold decrease in the apparent affinity (K_{a}) of the substrate androstenedione. In view of the complexity of the system (i.e. androstenedione uptake and enzymatic aromatization by intact cells), conclusive demonstration of a change in the K_{a} of aromatases awaits future purification of these enzymes.

It is generally accepted that the actions of estrogens are mediated through specific receptors in target tissues (37) and that for a given estrogen, a good correlation exists between receptor binding affinity and biologic potency (38). That the ovary is endowed with estrogen receptors was suggested by the demonstration of specific ovarian uptake of [1H]estradiol-17β (39) and the localization of estrogen receptors in the granulosa cells (20, 40). The order of stimulatory potencies of the native estrogens used in the present study is E2-17β = E1 > E3. This is in keeping with the fact that E2-17β and E1 are interconvertible in the granulosa cells by 17β-hydroxysteroid dehydrogenase and that E1 binds with a lower affinity to the ovarian estrogen receptors (40). Furthermore, E3-17α is also less effective than E2-17β. The findings that mestostrol and E2-17α are equipotent and that hexestrol is more potent than diethylstilbestrol are consistent with their relative binding affinities in mouse (41) and rat (42) uterine cytosols, respectively.

Based on the present findings, one is led to propose that estrogens, in addition to their diverse roles in stimulating granulosa cell maturation (9-27), may also be important in the regulation of ovarian estrogen production and the maintenance of the dominant ovarian follicles. The projected minimal effective dose of E2-17β capable of augmenting the aromatase activity is 3.7 x 10^{-10} M. This concentration is well within the reported range (up to 10^{-11} M) of E2-17β measured in the follicular fluid of rat (47) and human (48) preovulatory follicles, suggesting that the findings reported herein are of physiologic importance. Estrogens may act as an end product amplifier of aromatase activity to further enhance the synergistic effect of thecal androgens (8, 36). Steroids within the microenvironment of the ovarian follicle may activate intracellular autoregulatory positive feedback mechanisms to enhance the production of estrogens, leading to the preovulatory surge of estrogens.

It has been suggested that the selected follicle(s), by way of its estrogen production, reduces FSH levels thereby impeding the maturation of other follicles (49). However, it is not known how the selected follicle itself continues to mature in the face of declining FSH levels. The present finding suggests that intrafollicular estrogens can enhance the actions of FSH to stimulate aromatases. One "chosen" follicle(s) is producing a significant amount of estrogens, it has the capacity to produce more estrogens than neighboring follicles, and the selection of the dominant follicle(s) is secured. In contrast, follicles destined to undergo atresia may not be able to produce enough follicular estrogens to counteract the adverse effects of FSH deprivation. Future studies on the present...
granulosa cell cultures should provide important clues on the role of estrogens in intraovarian regulations. The granulosa cell cultures may also be a valuable in vitro model for studying the mechanism of action of estrogens.

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