Synergistic effects of steroids with FSH on folliculogenesis, steroidogenesis and FSH- and hCG-receptors in hypophysectomized mice

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Injection of ovine FSH ($4 \mu\text{g day}^{-1}$) for 4 days into hypophysectomized mice does not restore folliculogenesis to normal cyclic values. This may be due to insufficient production of oestradiol. The present study was designed to determine whether FSH- and LH-induced oestradiol was critical for growth and differentiation of follicles at all stages. Twelve days after hypophysectomy, mice were injected s.c. with 10, 50 or $250 \mu\text{g}$ oestradiol cyclopentylpropionate daily with or without ovine FSH ($4 \mu\text{g day}^{-1}$) for 1–4 days. One ovary from each animal was used for histology. From the second ovary, follicles were isolated at different stages and incubated with [^3H]thymidine for 3 h to determine the rate of DNA synthesis. Incubation medium and serum were used for steroid determinations. After oestradiol treatment alone, there was a dose-dependent response in serum oestradiol, but ovarian and uterine weights did not increase further with the increasing doses of oestradiol administered. This finding was consistent with an increase in the number of preantral follicles and small antral follicles but excluding the development of preovulatory follicles. Treatment with 10 and $50 \mu\text{g}$ of oestradiol cyclopentylpropionate did not prevent antral follicles from undergoing atresia. The higher dose ($250 \mu\text{g day}^{-1}$) of oestradiol cyclopentylpropionate delayed atresia of antral follicles and maintained more large healthy antral follicles. After concurrent injection of oestradiol and FSH, ovarian weight was 2–3 times greater than with either FSH or oestradiol alone; the number of follicles and follicular DNA synthesis at all stages of development increased without any signs of atresia; the *in vitro* accumulation of oestradiol also increased. Oestradiol alone induced FSH receptors in granulosa cells, but did not induce hCG receptors in any ovarian compartment; FSH alone induced FSH and hCG receptors in granulosa cells but not in thecal–interstitial tissues, whereas, oestradiol plus FSH enhanced FSH receptors in granulosa cells and LH/hCG receptors in granulosa and thecal–interstitial tissues. The synergistic effect of oestradiol with FSH was mimicked by the same dose of diethylstilboestrol, testosterone or dihydrotestosterone, but the latter steroids increased only the number of antral follicles, presumably because of their shorter half-lives. These results indicate that in mice oestradiol stimulates the growth of preantral and antral follicles and delays follicular atresia; oestrogens and androgens act synergistically with FSH to enhance follicular proliferation and differentiation, and prevent follicles from undergoing atresia.

Introduction

Pituitary gonadotrophins play a pivotal role in ovarian follicular development and steroidogenesis. Previous studies have demonstrated that hypophysectomy of adult mice decreases the number of follicles at all stages of development and the rate of follicular DNA synthesis (Wang and Greenwald, 1993a). Replacement of FSH in hypophysectomized mice stimulates the growth of preantral follicles to preovulatory stages and induces FSH and LH receptors only on granulosa cells. However, FSH alone does not induce LH receptors on thecal and interstitial

tissue, nor will it stimulate oestradiol production or prevent antral follicles from undergoing atresia (Wang and Greenwald, 1993b). Combined treatment with FSH and LH restores follicular structure and function to normal pro-oestrous values, in terms of the number of growing follicles at all stages of development, the rate of proliferation of granulosa cells, the distribution of FSH and LH receptors and oestradiol production (Wang and Greenwald, 1993b). This suggests that FSH and LH are both required for folliculogenesis, encompassing initiation, proliferation, differentiation and culminating in ovulation.

It is not known how LH acts on preantral follicles to synergize with the effects of FSH as there are no detectable LH receptors in granulosa cells of the early developmental follicles. On the basis of previous studies in rats, it is generally believed that oestrogens

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are essential for normal follicular development (Richards, 1980; Hsueh, 1986). In hypophysectomized immature rats, oestrogens enhance the proliferation of granulosa cells, diminish follicular atresia, augment the activities of steroidogenic enzymes, including aromatase activity, and induce their own receptors in granulosa cells and FSH and LH receptors (Goldenberg *et al.*, 1972; Kessel *et al.*, 1985; Hutz, 1989; Tonetta and diZerega, 1989). These results strongly indicate folliculotrophic effects of oestrogens on granulosa cells and growth of preantral and antral follicles. On the basis of these studies in rats, one hypothesis for the effect of LH on preantral follicles in hypophysectomized mice is that LH synergizes with FSH to stimulate oestrogen production; the latter, in turn, induces proliferation of granulosa cells and steroidogenesis, and subsequently increases the sensitivity of the follicle to further gonadotrophin stimulation.

The effects of steroids on follicular development and steroidogenesis in mice are unclear. There are only a few reports about the effects of oestrogen in immature mice, and it apparently exerts stimulatory or inhibitory effects on follicular development (Kent, 1973; Kim and Greenwald, 1987; Halling and Forsberg, 1990). The present study, using hypophysectomized cyclic mice as the experimental model, was undertaken to determine whether exogenous oestrogens and androgens are directly involved in (1) the preantral and early antral follicular development, by determining mean number and status of follicles as well as rate of DNA synthesis of isolated follicles at different developmental stages and (2) follicular differentiation, by measuring steroid concentrations in serum and the medium from incubated preovulatory follicles and localizing ovarian FSH- and LH/hCG-receptors.

A preliminary report of these results was presented at the 25th Annual Meeting of Society for the Study of Reproduction, North Carolina State University, Raleigh, July 1992.

Materials and Methods

Hypophysectomy and treatment

Adult female ICR mice (7–8 weeks old) were obtained from Harlan Co. (Madison, WI), and maintained under standard lighting conditions of 14 h light:10 h dark (lights on 06:00 h) at 20°C. Cyclic animals at random stages of the oestrous cycle were hypophysectomized by a parapharyngeal approach under Avertin (tribromoethyl alcohol, 0.4 ml of 2.5%) anaesthesia (day of hypophysectomy = day 0). After hypophysectomy, the drinking water was supplemented with 5% glucose. Completeness of hypophysectomy was assessed by previously described criteria (Smith and Tenney, 1979), and incompletely hypophysectomized animals were not included in the study. On day 12, animals were injected s.c. for 1–4 days with either (1) 20% polyvinyl pyrrolidone in saline or peanut oil (vehicle for steroids; hypophysectomized control group); or (2) NIDDK-oFSH-17 (4 µg day⁻¹) in polyvinyl pyrrolidone twice a day (a gift from the National Hormone and Pituitary Program, Baltimore, MD); or (3) different daily doses (10, 50 or 250 µg day⁻¹) of oestradiol cyclopentylpropionate (Upjohn, Kalamazoo, MI), an esterified oestrogen; or (4) oFSH-17 (4 µg day⁻¹) plus 250 µg day⁻¹ of oestradiol cyclopentylpropionate, diethylstilboestrol, dihydrotestosterone or testosterone; the last three were

purchased from Sigma (St Louis, MO). At 09:00 h after 1–4 days of treatment, animals were decapitated under ether anaesthesia and serum was saved for steroid assays. Ovaries and uteri were collected and weighed after drying on paper tissues.

Preparation for light and electron microscopy

One ovary from each animal was fixed in Bouin's fluid, overnight and sectioned serially at 7 µm. After routine histological processing, the sections were stained with haematoxylin–eosin. Follicles in the section in which the ovum and nucleolus were present were counted and classified as healthy or atretic. The presence of at least three pyknotic nuclei for stage 1–3 follicles and five pyknotic nuclei for stages 4–6 (defined below) in the largest cross-section of the follicle in which the nucleolus of the oocyte was present was considered as signs of early atresia; follicles with more than ten pyknotic nuclei were defined as late atretic follicles.

For preparation of specimens for electron microscopy, sliced ovarian tissues were fixed in 3% glutaraldehyde and post-fixed in 1% osmium tetroxide. The dehydrated tissues were then infiltrated and embedded in a 1:1 mixture of propylene oxide and epoxy resin. The trimmed tissues were sectioned using an LKB Nova ultramicrotome and the thin sections were stained by uranyl acetate and lead citrate solution. The stained sections were viewed and photographed with a JEOL JEM-100S transmission electron microscope.

Isolation of follicles and incubation of follicles with [³H]thymidine

The contralateral ovary to the one used for histology was cleaned; antral follicles (stages 4–6) were dissected out with watch-maker forceps; and preantral follicles (stages 1–3) were digested from the remaining ovarian tissue with a mixture of collagenase (Type 5) and DNase I in Krebs's-Ringer buffer containing 25 mmol Hepes l⁻¹ (Wang *et al.*, 1991). The isolated follicles (four replicates per stage) were incubated in 24-well culture clusters containing 700 µl buffer with 0.5% BSA for 3 h at 37°C in the presence of 0.5 µCi [³H]thymidine (Wang *et al.*, 1991). The number of follicles incubated for each stage was as follows: 50–70 stage 1 follicles (with a single layer of granulosa cells); 20–25 stage 2 follicles (two layers of granulosa cells); 20 stage 3 follicles (three or more layers of granulosa cells); 6 stage 4 follicles (incipient antrum); 5 stage 5 follicles (intermediate antrum) and 4 stage 6 (preovulatory follicles). The incubation was terminated by transferring follicles into 200 µl ice-cold DNA-assay buffer containing 50 µg unlabelled thymidine (Roy and Greenwald, 1986), and the follicles and the spent incubation medium were stored at –20°C until assayed for DNA content, radioactivity and steroids.

Follicular DNA content and incorporation of radionuclide into DNA were measured by fluorometry and scintillation counting, respectively (Roy and Greenwald, 1986). The results of incorporation of [³H]thymidine into follicles were expressed as fmol [³H]thymidine µg⁻¹ follicular DNA in 3 h.

Assay of steroids in serum and in incubation medium

Progesterone, androstenedione and oestradiol were assayed by radioimmunoassay as described by Terranova and Greenwald

(1978) and Wang and Greenwald (1993b). Non-specific binding and recoveries were performed for each assay. The lower limits of sensitivity in the assays for progesterone, androstenedione and oestradiol were 5, 2 and 2 pg per assay tube, respectively.

The spent medium (700 µl per well) from incubation of isolated follicles with [3 H]thymidine was used for steroid measurements. A preliminary experiment showed that after ether extraction and charcoal isolation of free and bound [3 H]-labelled hormones, free [3 H]thymidine in the incubation medium was removed completely and was within the background range of the radioimmunoassay. The incubation medium initially containing [3 H]thymidine was therefore used for steroid radioimmunoassays. Samples of 200 µl incubation medium were used to measure progesterone, androstenedione and oestradiol. About 400 µl serum was obtained from each mouse with 200 µl of serum used for the assay of oestradiol, about 100 µl for progesterone and 100 µl for androstenedione. All the incubation and serum samples were extracted with 2 ml of diethyl ether. Samples from each group were assayed at one time to avoid interassay variation. The results of *in vitro* steroid accumulations were expressed as pg per follicle in 3 h and serum steroids were expressed as pg ml $^{-1}$ serum.

Autoradiography for FSH and hCG receptors

Cleaned ovaries were frozen in liquid freon and stored at -80°C . A week before autoradiography, ovaries were sectioned at 8 µm in a cryostat at -20°C . The procedures for iodination, characterization and other preparation for autoradiography are described by Oxberry and Greenwald (1982) and Wang and Greenwald (1993a).

All hormones were supplied by the National Pituitary Hormone Program. Rat FSH I-8 (FSH bioactivity = $100 \times$ NIH FSH S-1 or 4714 iu mg $^{-1}$ of the 2nd IRP-HMG) and hCG CR-127 (14 900 iu mg $^{-1}$) were labelled with Na ^{125}I (IMS.300; Amersham, Arlington Heights, IL) by the lactoperoxidase method (Catt *et al.*, 1976) and characterized as described by Kim and Greenwald (1986) and Roy *et al.* (1987). The specific activities for the iodinated FSH and hCG were 27 and 31 µCi µg $^{-1}$; and the active fractions were 32 and 60%, respectively.

All labelled and unlabelled hormones were diluted with 0.05 mol Tris buffer l $^{-1}$ containing 0.01 mol CaCl $_2$ l $^{-1}$, 0.02 mol MgCl $_2$ l $^{-1}$ and 0.1% BSA. The sections were incubated at 37°C for 90 min with a 100 µl drop of 20 000 c.p.m. of ^{125}I -labelled rat rFSH or hCG. Nonspecific binding was determined by incubating sections in the presence of a 500-fold excess of unlabelled homologous hormone in addition to labelled hormone. Specific binding was determined by incubating sections with ^{125}I -labelled hormone plus a 500-fold excess of one of the unlabelled heterologous hormones. After incubation, the sections were washed in three changes of phosphate buffered saline at 4°C and postfixed for 10 min in 3% glutaraldehyde-phosphate buffered saline. After rinsing and air-drying, the sections were coated with Kodak NTB-2 liquid emulsion, exposed at 4°C for two weeks for hCG binding or three weeks for FSH binding, developed in Dektol (Kodak) and stained with haematoxylin.

Fig. 1. Effects of replacement of FSH and oestradiol on uterine and ovarian weights in hypophysectomized mice. (a): uterine weight; (b): ovarian weight. (○) 250 µg oestradiol day $^{-1}$, (●) 4 µg FSH day $^{-1}$ or (■) FSH plus oestradiol were injected beginning on day 12 after hypophysectomy for 4 days. The mean ($\pm$ SEM) were based on 25 g of body weight from four to six animals in each group. *The weight of these groups were significantly increased ($P < 0.05$) compared with (Δ) hypophysectomized controls on the same day of treatment.

Statistical analysis

One-factor ANOVA followed by the Fisher protected least significant difference or Duncan's new multiple range test were used for most data analysis and two-factor ANOVA followed by the least squares means test of T was applied for multiple comparisons for the data in Fig. 1 by using the Super ANOVA program (Abacus Concepts, Inc. Berkeley, CA) on a Macintosh computer. Values were considered statistically different when significance was at least $P < 0.05$.

Results

Ovarian and uterine weight

After 4 days of injections of different doses (10, 50 or 250 µg day $^{-1}$) of oestradiol cyclopentylpropionate, serum oestradiol increased with the increasing doses administered, from about 10 pg ml $^{-1}$ in the hypophysectomized control to 7830 pg ml $^{-1}$ for the oestradiol group with highest dose (250 µg). Ovarian and uterine weights increased significantly even by day 3 of the 10 µg oestradiol injections ($P < 0.05$), and saturation was reached with higher doses. However, neither ovarian nor uterine weights were restored to pro-oestrous values. When oestradiol (250 µg day $^{-1}$) was combined with FSH (4 µg day $^{-1}$) and administered to hypophysectomized mice for 1–4 days, the rate and the maximum increase in uterine weight was not different from those of mice receiving oestradiol alone, but was much higher than those of the FSH-alone group (Fig. 1a).

Fig. 2. Effects of replacement of FSH and/or oestradiol cyclopentylpropionate (ECP), diethylstilboestrol (DES), dihydrotestosterone (DHT), testosterone (T) on concentrations of (a) progesterone, (b) androstenedione and (c) oestradiol in serum (□) and in incubation medium (■) for isolated preovulatory (stage 6) follicles in HX mice. Note that there were no stage 6 follicles in hypophysectomized controls (HX) nor in the HX + ECP group (no); ^asteroid concentrations were not detectable in the marked groups; ^{b-d}values with different letters were significantly different ($P < 0.05$) among the groups for steroid accumulation in follicular incubation medium; ^{x-z}values with different letters were significantly different ($P < 0.05$) among the groups for serum steroid concentrations.

However, ovarian weight in the oestradiol plus FSH group increased significantly ($P < 0.05$) compared with those of groups treated with FSH or oestradiol alone (Fig. 1b). Diethylstilboestrol, dihydrotestosterone or testosterone (250 µg daily) also acted synergistically with FSH to increase ovarian and uterine weight (data not shown).

Steroid concentrations after treatment for 4 days

No preovulatory follicles (stage 6) were present in hypophysectomized controls or in the group receiving oestradiol alone; there was no detectable accumulation of steroids in the incubation medium for follicles smaller than stage 5 (Fig. 2). Serum progesterone and androstenedione were not detectable in hypophysectomized controls and the concentration of serum oestradiol was low (about 10 pg ml⁻¹) in hypophysectomized controls and in groups receiving FSH alone, diethylstilboestrol plus FSH or dihydrotestosterone plus FSH. This might be the result of oestradiol-antibody crossreacting with oestrogen

analogues, such as equol (Barkley *et al.*, 1985). However, exogenous oestradiol (250 µg day⁻¹) not only led to high concentrations of serum oestradiol, but also caused high concentrations of serum progesterone and androstenedione. As reported previously (Wang and Greenwald, 1993b), treatment of hypophysectomized mice with FSH alone did not increase serum concentrations of progesterone, androstenedione and oestradiol. In contrast to groups receiving oestradiol or FSH alone, the groups receiving FSH with oestradiol, diethylstilboestrol, dihydrotestosterone or testosterone secreted progesterone, androstenedione and oestradiol into serum and into the incubation medium at different rates (Fig. 2).

Follicular development

Treatment with oestradiol alone at lower doses (10 or 50 µg day⁻¹) for 4 days in hypophysectomized mice increased the number of healthy follicles at all stages of development (stages 1–5) compared with the hypophysectomized control group, but did not prevent follicles from becoming atretic, nor support follicles growing to the preovulatory stage (stage 6) (Table 1). A higher dose of oestradiol (250 µg day⁻¹) significantly increased the number of stage 1–5 follicles after 2 days of treatment (data not shown), but did not increase the number of preantral follicles (stages 1–3) significantly after 4 days of treatment, although the number of healthy medium-sized follicles (stages 4–5) was greater than in hypophysectomized controls. In the hypophysectomized group receiving 250 µg oestradiol, about 60–70% atretic follicles were at an early atretic stage in which degenerating granulosa cells showed nuclear compaction in uniformly dense masses but not breakdown, fewer mitochondria, swollen rough endoplasmic reticulum and cytoplasmic condensation (Fig. 3a). In the remaining healthy granulosa cells of the same follicle, there were some focal sites of degeneration. These early atretic signs persisted for the 4 days of treatment with 250 µg oestradiol day⁻¹. Treatment with 10 and 50 µg oestradiol day⁻¹ or with FSH alone did not prevent follicles from undergoing atresia; 90% of the atretic follicles were at late stages (Fig. 3b). There were many pyknotic cells evenly scattered throughout the mural granulosa cells. The degenerating cells contained a broken pyknotic nucleus, electron-dense lipid droplets and unclear cytoplasmic organelles. A very low ratio of cytoplasm to nucleus was observed even in the relatively healthy cells in the advanced atretic follicles (Fig. 3b).

The combined injection of oestradiol and FSH in hypophysectomized mice significantly increased the number of follicles at all stages of development (stages 1–6) and caused superfollicular growth without any signs of atresia (Table 1). Diethylstilboestrol acted synergistically with FSH on folliculogenesis in the same way as oestradiol but was less potent because it has a shorter half-life than does oestradiol. In addition, androgens (dihydrotestosterone and testosterone), when substituted for oestrogens, enhanced the number of middle and large-sized follicles without increasing the number of small follicles. In the hypophysectomized group receiving testosterone, fewer follicles underwent atresia. Again, the shorter half-lives of the androgens compared with oestradiol probably accounts for their inability to stimulate smaller follicles.

Table 1. Effects of FSH, steroid replacement or both treatments on numbers of healthy and atretic follicles per mouse ovary (mean $\pm$ SEM) at different developmental stages on day 16 after hypophysectomy (HX)

Treatment ^a	Stage of follicles					
	1	2	3	4	5	6
HX control	83 $\pm$ 18	68 $\pm$ 19	39 $\pm$ 4	7 $\pm$ 1 (2) [†]	4 $\pm$ 0.3 (14)	None
HX + ECP						
10 μ g day ⁻¹	176 $\pm$ 27*	125 $\pm$ 21*	61 $\pm$ 5*	26 $\pm$ 7* (20)	9 $\pm$ 2* (18)	None
50 μ g day ⁻¹	164 $\pm$ 28*	121 $\pm$ 18*	54 $\pm$ 5*	27 $\pm$ 7* (9)	7 $\pm$ 2 (17)	None
250 μ g day ⁻¹	109 $\pm$ 7	88 $\pm$ 12	58 $\pm$ 4*	29 $\pm$ 5* (3)	20 $\pm$ 2* (13)	None
HX + FSH	148 $\pm$ 20*	130 $\pm$ 17*	71 $\pm$ 8*	17 $\pm$ 2* (6)	13 $\pm$ 2* (13)	6 $\pm$ 2* (10)
HX + FSH + ECP	180 $\pm$ 12*	126 $\pm$ 14*	91 $\pm$ 6*	38 $\pm$ 11*	37 $\pm$ 3*	73 $\pm$ 13*
HX + FSH + DES	160 $\pm$ 27*	76 $\pm$ 10	58 $\pm$ 8	18 $\pm$ 2*	12 $\pm$ 2*	33 $\pm$ 7*
HX + FSH + DHT	113 $\pm$ 18	72 $\pm$ 10	64 $\pm$ 11*	27 $\pm$ 3*	27 $\pm$ 6*	49 $\pm$ 9*
HX + FSH + T	86 $\pm$ 9	64 $\pm$ 6	56 $\pm$ 6*	26 $\pm$ 5* (4)	22 $\pm$ 3* (3)	44 $\pm$ 5* (6)

^as.c. Injection of FSH (4 μ g day⁻¹) in polyvinyl pyrrolidone twice a day and s.c. injection (250 μ g day⁻¹) of oestradiol cyclopentylpropionate (ECP), diethylstilboestrol (DES), dihydrotestosterone (DHT) or testosterone (T) in peanut oil once a day for 4 days beginning at day 12 after hypophysectomy.

[†]Values in parentheses are the mean number of atretic follicles at that stage.

* $P < 0.05$, comparing different treatment groups with the hypophysectomized control group within that developmental stage ($n = 4-8$).

Incorporation of [³H]thymidine

The *in vitro* incorporation of [³H]thymidine into DNA of isolated follicles at different stages was used to evaluate the rate of follicular DNA synthesis (Table 2). The DNA content of follicles from hypophysectomized mice at most stages was unchanged from the content in cyclic mice (data not shown, see Wang *et al.*, 1991), indicating that the cell mass in each follicle was similar before and after hormonal treatment. However, for the hypophysectomized group receiving FSH plus oestradiol, preovulatory follicles were induced after only 1 day of treatment and the DNA content of preovulatory follicles (stage 6) increased from 150 $\pm$ 14 ng per follicle after 1 day of treatment to 462 $\pm$ 24 ng per follicle after 4 days. This finding indicated that the preovulatory follicles grew continuously and atresia had been eliminated. After 3–4 days of treatment, the preovulatory follicles were larger than those from normal pro-oestrous mice. The rate of DNA synthesis was therefore expressed as fmol [³H]thymidine incorporated μ g⁻¹ DNA rather than per follicle.

Concomitant with the increase in the number of follicles, there was increased [³H]thymidine incorporation into preantral (stages 1–3) and antral (stages 4–6) follicles in the groups receiving FSH plus oestrogen and FSH plus diethylstilboestrol (Table 2). In the groups receiving FSH plus dihydrotestosterone or FSH plus testosterone, [³H]thymidine incorporation increased only in antral follicles (stages 4–6). For groups treated with 10 or 50 μ g oestradiol alone, although the number of follicles increased in most stages (Table 1), the rate of DNA synthesis increased only in follicles at stages 2 and 4; incorporation of [³H]thymidine into follicles at stages 2–4 increased in the group treated with 250 μ g oestradiol. Regardless of the dose of oestradiol, there was no increased incorporation of [³H]thymidine for follicles at stage 5, which was the last stage of follicular development attained in the absence of gonadotrophins, and is associated with reduced DNA synthesis and follicular atresia. The best result in terms of DNA synthesis was achieved by

treatment with FSH plus oestradiol, in which increased incorporation of [³H]thymidine occurred in all stages of follicular development.

FSH- and hCG-receptors

Oestradiol induced FSH receptors in the granulosa cells of follicles larger than stage 3 (Fig. 4a, b), as did FSH alone (Fig. 4c); FSH plus oestradiol resulted in a greater density of grains in granulosa cells compared with either FSH or oestradiol alone (Fig. 4d). For hCG receptors, trace amounts of [¹²⁵I]-labelled hCG binding was limited to the interstitium after oestradiol treatment (Fig. 5a), similar to the hypophysectomized control (not shown). As reported by Wang and Greenwald (1993b), treatment with FSH alone induced hCG receptors in granulosa cells only in follicles at stages 5 and 6 without thecal and interstitial binding sites (Fig. 5b). However, combined treatment with oestradiol and FSH induced hCG receptors not only in granulosa cells but also in the theca and interstitium (Fig. 5c, d), comparable to that in pro-oestrous mice and hypophysectomized mice treated with FSH plus LH. It is noteworthy that treatment with oestradiol plus FSH induced hCG receptors in granulosa cells, even in follicles at stage 3–4; this was not observed in pro-oestrous mice (Wang and Greenwald, 1993a).

Discussion

Hypophysectomized mice were used in this study to evaluate the actions of certain steroids, either alone or in combination with FSH, on follicular development and differentiation. The results demonstrate that oestrogens alone directly stimulate growth of follicles, but do not enhance follicular differentiation; however, oestrogen plus FSH stimulates both follicular growth and differentiation. The mechanisms for the synergistic effects

Fig. 3. Photomicrographs of follicles at stage 5 showing different degrees of atresia after (a) oestradiol or (b) FSH replacement in hypophysectomized mice. Oestradiol ($250 \mu\text{g day}^{-1}$) or FSH ($4 \mu\text{g day}^{-1}$) was injected for 4 days beginning on day 12 after hypophysectomy and the mice were killed on day 16. Insets of each panel are light micrographs. RER: rough endoplasmic reticulum; M: mitochondria; LD: lipid droplets; FD: focal sites of degeneration. Arrowheads point to mitoses. Note: there are also pyknotic nuclei (dark staining) scattered among healthy cells. Scale bars = (a) $5 \mu\text{m}$, (b) $10 \mu\text{m}$, inserts: $20 \mu\text{m}$.

of oestrogens with FSH involve enhancement of steroid production, especially oestradiol, and induction of ovarian FSH and LH/hCG receptors.

Williams (1940) and Pencharz (1940) were the first to demonstrate that diethylstilboestrol increased ovarian weight in hypophysectomized immature rats. The optimal stimulatory dose for this effect is 1–2 mg for 3–4 days (established by Payne and Hellbaum, 1955). On the basis of this and other studies (Goldenberg *et al.*, 1972; Kim and Greenwald, 1987), the doses of oestrogen given to hypophysectomized mice were

10–250 $\mu\text{g day}^{-1}$ for 1–4 days; these are lower than the optimal dose because of the relative longer half-life of oestradiol cyclopentylpropionate (72 h in ovariectomized hamsters; Greenwald, 1975). After 4 days of treatment with oestradiol, serum oestradiol concentration was as high as 8000 pg ml^{-1} compared with 35 pg ml^{-1} in intact pro-oestrous mice (Wang and Greenwald, 1993b). A pharmacological dose of oestradiol was therefore used in an attempt to achieve significantly higher oestradiol concentrations in follicular fluid than in the serum (about 500-fold difference in rats; Goff and Henderson, 1979).

Table 2. Effects of FSH and steroids on the incorporation of [³H]thymidine into follicular DNA of hypophysectomized mice ([³H]thymidine fmol µg DNA⁻¹, mean ± SEM)

Treatment ^a	Stage of isolated follicles					
	1	2	3	4	5	6
HX control	65.9 ± 7.6	111.0 ± 15.4	160.7 ± 10.8	117.8 ± 9.6	157.0 ± 17.6	None
HX + ECP (10 µg)	71.0 ± 8.5	180.4 ± 23.2*	165.7 ± 4.8	217.9 ± 13.5*	144.5 ± 17.6	None
HX + ECP (50 µg)	86.2 ± 9.0	158.2 ± 21.8*	183.5 ± 34.3	212.1 ± 15.5*	162.1 ± 7.5	None
HX + ECP (250 µg)	105.9 ± 6.7	227.1 ± 7.1*	300.7 ± 44.2*	307.4 ± 9.3*	193.7 ± 18.2	None
HX + FSH	128.0 ± 27.6*	152.3 ± 8.7	194.7 ± 19.3	115.8 ± 21.1	181.0 ± 15.1	110.5 ± 9.1*
HX + FSH + ECP	154.9 ± 20.2*	237.7 ± 9.2*	310.3 ± 26.9*	460.1 ± 31.3*	457.6 ± 21.0*	145.9 ± 14.9*
HX + FSH + DES	195.0 ± 29.3*	253.2 ± 22.6*	261.5 ± 27.8*	362.5 ± 48.9*	301.4 ± 24.2*	117.7 ± 4.7*
HX + FSH + DHT	111.6 ± 11.5	135.7 ± 5.6	154.8 ± 4.2	533.2 ± 60.8*	402.2 ± 18.1*	153.0 ± 8.9*
HX + FSH + T	74.6 ± 14.3	137.6 ± 4.9	160.6 ± 20.2	361.3 ± 49.5*	460.2 ± 23.7*	273.3 ± 12.2*

^aDifferent daily doses of oestradiol cyclopentylpropionate (ECP) in peanut oil, as indicated in the table, were injected s.c. once a day for 4 days beginning on day 12 after hypophysectomy (HX). Combinations of FSH (4 µg day⁻¹) and 250 µg day⁻¹ of ECP, diethylstilboestrol (DES), dihydrotestosterone (DHT), or testosterone (T) were also given for 4 days and the animals were killed on day 16 after hypophysectomy.

**P* < 0.05, comparing different treatment groups with the hypophysectomized control group within that developmental stage, *n* = 4.

Fig. 4. Topical autoradiographs showing the effects of oestradiol replacement with or without FSH on intraovarian distribution of binding sites for [¹²⁵I]FSH. The panels represent ovarian sections from hypophysectomized mice after treatment for 4 days. (a) hypophysectomized control: no significant binding grains in granulosa layers of follicles at stage 3 and 4; (b) 250 µg oestradiol cyclopentylpropionate day⁻¹, (c) 4 µg FSH day⁻¹ or (d) oestradiol plus FSH. (b–d) show increasing binding density for FSH receptors in granulosa cells of preantral and antral follicles. Numbers in the figures represent the stage of follicles i.e. '3' is a stage 3 follicle. Scale bar = 70 µm for all parts of this figure.

Unexpectedly, a dose of oestradiol as low as 10 µg day⁻¹ for 4 days increased ovarian and uterine weights in hypophysectomized mice to the same degree as 250 µg oestradiol,

although serum oestradiol was only 240 pg ml⁻¹. This correlates with the response of follicular development; i.e. 10 or 50 µg oestradiol increased the number of follicles at all stages of

Fig. 5. Typical autoradiographs of the effects of oestradiol replacement with or without FSH on intra-ovarian distribution of binding sites for [125 I]hCG. The panels represent ovarian sections from hypophysectomized mice after injection of (a) 250 μ g oestradiol cyclopentylpropionate day $^{-1}$ for 4 days: trace amounts of binding grains appear only in the interstitium; (b) 4 μ g FSH day $^{-1}$ for 4 days: binding activity appears only in mural granulosa layers of large antral follicles but not in thecal and interstitial tissues; (c) oestradiol plus FSH for 3 days; and (d) oestradiol plus FSH for 4 days. Treatment with oestradiol plus FSH induced hCG receptors in granulosa and thecal/interstitial regions. Numbers in the figures represent the stage of follicles i.e. '4' is a stage 4 follicle. Arrowheads point to thecal cells. Scale bar = 70 μ m for all parts of this figure.

development except for preovulatory follicles, but the rate of DNA synthesis increased only in follicles at stages 2 and 4. This finding is consistent with the morphological changes of pre-antral follicles, in which there is a large oocyte surrounded by few layers of granulosa cells (not shown), comparable to the status in hypophysectomized control animals (Wang and Greenwald, 1993a). This implies that although the low doses of oestradiol (10 or 50 μ g day $^{-1}$) enhanced follicular development, the rate of proliferation of granulosa cells is slower than the growth of the oocyte; the transition time from one stage to the next may be longer, resulting in accumulation of more follicles at each stage. When oestradiol plus FSH or FSH plus LH are administered, follicular recruitment and proliferation are stimulated (Wang and Greenwald, 1993b).

A higher dose (250 μ g) of oestradiol stimulated the proliferation of granulosa cells, as shown by the increased rate of DNA

synthesis and the large number of medium follicles with a large granulosa cell mass and small antral cavity; most of these follicles were healthy or at early stages of atresia. This result is comparable to a previous study of oestrogen administration to immature mice, although healthy and atretic follicles were not distinguished (Kim and Greenwald, 1987). In rats, high concentrations of oestrogens in follicular fluid also exert a direct anti-atretic effect (Harman *et al.*, 1975; Hsueh, 1986). However, the exact mechanism involved in stimulation of follicular growth and reduction of follicular atresia remains uncertain. This type of cell degeneration in follicular atresia is known as apoptosis (O'Shea *et al.*, 1978). Experiments using RNA and protein synthesis inhibitors have shown suppression or induction of apoptosis, implying the presence of either intracellular repressors or inducers of apoptosis, which may be steroid or peptide hormones (Gerschenson and Rotello, 1992). For

example, removal of androgens results in apoptosis in the prostatic epithelium (Kyrianiou *et al.*, 1990); progesterone withdrawal elicits apoptosis in uterine epithelial cells (Rotello *et al.*, 1991a) and transforming-growth factor- β 1 induces apoptosis in primary cultures of uterine epithelial cells (Rotello *et al.*, 1991b). Oestrogen may be one of the candidates regulating follicular atresia since oestradiol slows down the process of follicular atresia in hypophysectomized mice for the first 4 days of treatment; advanced atresia, however, has been observed when oestrogen treatment is continued for more than four days in immature hypophysectomized diethylstilboestrol-treated rats (Sadkhanloo *et al.*, 1987).

A combination of oestradiol and FSH results in superfollicular development in contrast to combinations of FSH plus LH; the latter treatment induces about the same number of preovulatory follicles as in normal cyclic mice (Halpin and Charlton, 1988; Wang and Greenwald, 1993b). The superfollicular development may be attributed to the high concentration of oestrogen, since the rate of DNA synthesis in follicles increases at all stages of development and none of the follicles becomes atretic. The mechanism for the synergistic effect of oestradiol with FSH has been investigated in rats and reviewed by Richards (1987) and Richards and Hedin (1988). Studies suggest that oestrogen increases FSH-induced adenylyl cyclase and cAMP formation (Richards *et al.*, 1979; Knecht *et al.*, 1984) and cAMP-dependent protein kinases, which in turn phosphorylate the oestradiol receptor in granulosa cells of large antral follicles in rats (Richards, 1975; Richards and Rolfes, 1980; Green *et al.*, 1986). However, the existence of oestradiol receptors in mouse pre-antral follicles needs to be established to determine whether the effect of oestradiol on these follicles is directly mediated by receptors or indirectly mediated by growth factors, such as, transforming-growth factor- β (Bendell and Dorrington, 1991).

Another mechanism by which oestrogen increases the response of granulosa cells to gonadotrophins is to induce FSH receptors on granulosa cells of follicles beyond stage 3 (late preantral follicles), as reported in hypogonadal mice (Charlton *et al.*, 1982) and hypophysectomized immature rats, where it is cAMP-mediated (Goldenberg *et al.*, 1972; Knecht *et al.*, 1984). Treatment with oestradiol alone does not result in an increase in LH/hCG receptors on granulosa cells and thecal-interstitial tissues in rats (Rao *et al.*, 1991) but, in hypogonadal mice (Charlton *et al.*, 1982), oestrogen implanted for 5 days can induce formation of large antral follicles and hCG labelling on granulosa cells and interstitial and thecal cells. The different response to oestrogen between hypophysectomized and hypogonadal mice may be due to oestrogen acting directly in the pituitary to stimulate gonadotrophin secretion. In hypophysectomized mice, treatment with FSH alone induces hCG receptors on granulosa cells of large antral follicles, but not on the theca nor the interstitium (Wang and Greenwald, 1993b). Combined treatment with oestradiol and FSH induces hCG receptors on granulosa and thecal-interstitial regions. hCG receptors are observed even on stage 4 follicles (early antral stage); this is not the case in normal, cyclic mice (Wang and Greenwald, 1993a). Thus, oestradiol acts synergistically with FSH to enhance the differentiation of granulosa cells to express and synthesize LH/hCG receptors at an earlier stage than normal (Kessel *et al.*, 1985; Segaloff *et al.*, 1990).

As reported previously, treatment with FSH alone did not induce oestradiol production (Schoot *et al.*, 1992; Wang and Greenwald, 1993b). This may be attributable not to lower aromatase activity (Mannaerts *et al.*, 1991) but to reduced amounts of precursors of oestradiol, progesterone and androstenedione as a consequence of undeveloped or undifferentiated thecal cells in the absence of LH stimulation. Oestradiol combined with FSH increases progesterone, androstenedione and oestradiol in serum and in the *in vitro* medium. This finding implies that oestrogen and FSH are both required for the entire steroid pathway in follicles, as in rats (Fitzpatrick and Richards, 1991). Oestradiol cyclopentylpropionate dissociates *in vivo* into oestradiol (Greenwald, 1975), which was measurable by radio-immunoassay. It is possible that the accumulation of oestradiol in medium represents storage in follicles of oestradiol derived from injected oestradiol cyclopentylpropionate. However, diethylstilboestrol combined with FSH also showed the same effects as oestradiol cyclopentylpropionate on the *in vitro* accumulation of progesterone, androstenedione and oestradiol. Since diethylstilboestrol does not crossreact with the oestradiol radioimmunoassay, the possibility of contamination can be ruled out.

The present study also demonstrates that concurrent treatment with FSH and androgens, dihydrotestosterone (non-aromatizable) and testosterone stimulates only antral follicle growth (but not preantral follicles), prevents atresia and stimulates progesterone, androstenedione and oestradiol production. These results are consistent with the conclusion that androgens enhance FSH-induced aromatase activity and gene expression in cultured rat granulosa cells (Daniel and Armstrong, 1980; Fitzpatrick and Richards, 1991), but is not consistent with the conclusion that dihydrotestosterone reduces ovulation rate by decreasing the number of granulosa cells of follicles and by altering the ability of the cells in immature PMSG-treated rats to synthesize oestrogen (Conway *et al.*, 1990). These studies suggest that the ovarian actions of androgens are diverse. Generally, in the absence of FSH, androgens exert mainly negative effects (Conway *et al.*, 1990), whereas in the presence of FSH, androgens augment FSH stimulation of progesterone and oestradiol biosynthesis by enhancing aromatase activity in rat granulosa cells (Daniel and Armstrong, 1980; Conway and Mills, 1991). Thus, LH regulation of thecal cell production of androgens in rats and mice plays a key role in determining ultimately which follicles continue to respond to FSH and undergo differentiation.

It is not surprising that exogenous testosterone enhances the production of oestradiol because it can be converted to oestradiol by aromatase, induced in granulosa cells by FSH (Mannaerts *et al.*, 1991). Dihydrotestosterone cannot be converted to oestrogens. However, it directly stimulates the gene expression of aromatase as effectively as oestradiol and testosterone (Fitzpatrick and Richards, 1991). This finding suggests that during follicular development, theca-derived androgens enhance the actions of FSH and serve as stimulators and substrates for aromatase. The induction of oestradiol production by androgens also explains why superfollicular development occurs without any signs of atresia when FSH is combined not only with LH and oestrogens but also with androgens. In an *in vitro* culture system, testosterone acting synergistically with FSH was more effective in stimulating oestradiol production

than was dihydrotestosterone (Daniel and Armstrong, 1980). However, the ovarian actions of testosterone in the present study are not as effective as oestradiol cyclopentylpropionate and dihydrotestosterone in stimulating production of progesterone, androstenedione and oestradiol and preventing atresia; presumably because testosterone administered *in vivo* can be converted to a variety of metabolites in extra-ovarian tissues. In addition, dihydrotestosterone has a higher affinity for the androgen receptor than does testosterone in the rat prostate (Lamb *et al.*, 1992); this provides another explanation for the observation that dihydrotestosterone is more effective than testosterone in stimulating folliculogenesis and steroidogenesis.

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