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Effect of diethylstilbesterol and prolactin on the induction of follicle stimulating hormone receptors in immature and cycling rats

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Abstract. Induction of follicle stimulating hormone receptor in the granulosa cells of intact immature rat ovary by diethylstilbesterol, an estrogen, has been studied.

A single injection of 4 mg of diethylstilbesterol produced 72 h later a 3-fold increase in follicle stimulating hormone receptor concentration as monitored by [^{125}I]-oFSH binding to isolated cells. The newly induced receptors were kinetically indistinguishable from the pre-existing ones, as determined by Lineweaver-Burk plot of the binding data. The induced receptors were functional as evidenced by increased ability of the granulosa cells to incorporate [^{3}H]-leucine into cellular proteins.

Neutralization of endogenous follicle stimulating hormone and luteinizing hormone by administering specific antisera had no effect on the ability of diethylstilbesterol to induce follicle stimulating hormone receptors, whereas blockade of endogenous prolactin secretion by ergobromocryptin administration significantly inhibited (~ 30 %) the response to diethyl-stilbesterol; this inhibition could be completely relieved by ovine prolactin treatment. However, ovine prolactin at the dose tried did not by itself enhance follicle stimulating hormone receptor level.

Administration of ergobromocryptin to adult cycling rats at noon of proestrus brought about as measured on diestrus II, (a) a reduction of both follicle stimulating hormone (~ 30 %) and luteinizing hormone (~ 45 %) receptor concentration in granulosa cells, (b) a drastic reduction in the ovarian tissue estradiol with no change in tissue progesterone and (c) reduction in the ability of isolated granulosa cells to convert testosterone to estradiol in response to follicle stimulating hormone. Ergobromocryptin treatment affected only prolactin and not follicle stimulating hormone or luteinizing hormone surges on the proestrus evening. Treatment of rats with ergobromocryptin at proestrus noon followed by an injection of ovine prolactin (1 mg) at 1700 h of the same day completely reversed the ergobromocryptin induced reduction in ovarian tissue estradiol as well as the aromatase activity of the granulosa cells on diestrus II, thus suggesting a role for proestrus prolactin surge in the follicular maturation process.

Keywords. Gonadotropins; receptor induction; prolactin; estrogen; follicle.

Introduction

Over the last several years considerable interest has been generated to study the mechanism of induction of gonadotropin receptors. The receptors for follicle

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Abbreviations used: FSH, Follicle stimulating hormone; LH, luteinizing hormone; DES, diethylstilbesterol; EBC, ergobromocryptin; hCG, human chorionic gonadotropin; PMSG, pregnant mare serum gonado-tropin; Prl, prolactin.

stimulating hormone (FSH) have been demonstrated to be located exclusively in the granulosa cells of the ovary (Midgley, 1973; Zeleznik *et al.,* 1974; Nimrod *et al.,* 1976), but the factor responsible for the induction of FSH receptors in these cells is still not clear. Goldenberg *et al.* (1972) demonstrated increased ovarian uptake of [^{3}H]-FSH *in vivo* upon diethylstilbesterol (DES) administration. This was due to an increase in the number of granulosa cells with no apparent change in FSH binding sites per cell and the binding association constant (Louvet and Vaitukaitis, 1976). It was concluded (Richards, 1979) that the increased responsiveness of the granulosa cells of hypophysectomized immature rats, pretreated with estrogen, to FSH was not related to changes in the FSH binding sites per cell, but that an estradiol treatment for 12-24h was facilitatory in FSH inducing its own receptor (Richards *et al.,* 1976).

In the above studies the dose and duration of estrogen treatment was large and prolonged. Further, most studies involved working with hypophysectomized animals. A reinvestigation of some aspects of this problem using intact immature rats and a different dose schedule of estrogen was therefore felt desirable.

In the present study we essentially describe the intrinsic ability of estrogen to induce FSH receptors, the kinetic analysis and functionality of these receptors have also been made. In the course of these studies the involvement of prolactin (Prl) in the inductive process both in estrogen treated immature rat model as well as in the adult cycling rat model has been uncovered.

Materials and methods

Hormones, antisera and chemicals

Purified ovine follitropin S1528 C_2R (~ 50 × NIH FSH S_1) was a kind gift from Dr. M. R. Sairam, Canada. Human chorionic gonadotropin (hCG) (CR 123) was obtained from Dr. R. Canfeild through NIAMDD, Bethesda, USA. Ovine prolactin (NIH PS 13), ovine FSH (NIH oFSH SI 5) and radioimmunoassay kits for estimation of rFSH, rLH and rPrl were obtained from NIAMDD, Bethesda, USA. Ergobromocryptin (EBC) was obtained from Sandoz, Basle, Switzerland. The following chemicals were purchased from British Drug House Chemicals, UK and were of AnalaR grade: acetaldehyde, glucose and chloramine-T. Diphenylamine, glacial acetic acid, propylene glycol were obtained from British Drug House Chemicals, Bombay. Estradiol-17β and progesterone were obtained from Steraloids, Inc., Wilton, New Haven, USA. DES, β-mercaptoethylamine hydrochloride, bovine serum albumin were supplied by Sigma Chemical Co., St. Louis, Missouri, USA. Carrier free Na[^{125}I], (2,4,6,7-^{3} H) estradiol-17β, (1,2,6,7-^{3}H) progesterone and L (4,5-^{3} H) leucine were obtained from Radiochemical Centre, Amersham, UK.

Production and characterization of monkey antiserum to oFSH and oLH was carried out as described earlier by Sheela Rani and Moudgal (1977). To determine the amount of rat FSHRP_1 and rat luteinizing hormone (LH)RP_2 (NIAMDD reference preparations) needed to saturate a known volume of antiserum to FSH and LH respectively, a titration using [^{125}I]-labelled rat FSH and LH was performed. Graded doses of rFSH RP_1 were incubated with 0·01 μl of the antiserum to FSH for 12 h followed by incubation with [^{125}I]-rat FSH for 12 h. Sufficient normal monkey serum

and goat antiserum to monkey γ-globulin were added and incubation continued for 12h, the precipitate obtained was counted for bound radioactivity. All incubations were carried out at 37°C. 5 μg of rat FSH RP_1 was found to completely saturate 0·01 μl of the antiserum. Using [^{125}I] -rat LH and rat LH RP_2 reference preparation, the ability of 0·02 μ1 of the LH antiserum to bind rat LH was determined. 500 ng of rat LH R P $_2$ (61 times purer than rat LH RP_1) could completely be neutralized by 0·02 μl of the antiserum to LH. Further, the presence of FSH and LH antibody in the serum samples obtained at the time of autopsy from animals treated with 300 μl of the FSH and LH antiserum 3 days earlier, was indicated by the ability of 100μl of serum to consistently bind 70 and 60 % [^{125}I] -iodo rat FSH and LH respectively. Undiluted FSH and LH antisera (100 μl) bound 80 and 70% radiolabeled rat FSH and LH respectively. Progesterone antiserum was raised in a rabbit as previously described (Mukku and Moudgal., 1975). Antiserum specific to estradiol-17β was obtained as a kind gift from Dr. P. N. Rao, South West Foundation, Texas, USA. It cross-reacted with estriol to the extent of 0·6 %, but showed no cross-reactivity with estrone or C-19 steroids.

Animals and treatment

Two month and twenty-one-day old female albino rats of our Institute colony originally derived from the Wistar strain were maintained under a light/dark schedule of 14:10h. They were fed a standard pellet diet (Hindustan Lever Ltd., Bombay) and given water *ad libitum.* Adult cycling rats were checked for regularity of cycle for at least four consecutive cycles by observing vaginal smears. Those animals which exhibited at least three regular cycles were used in the experiments. DES, progesterone (in 100 μl of propylene glycol) and EBC (in 100 μl of 50% ethanol) were injected s.c. oFSH (NIH o FSH S15), oPrl (100μl; dissolved in minimal vol. of 0·1 N NaOH and diluted in saline) and monkey antiserum to FSH and LH were injected intraperitoneally.

Iodination of hormones

Iodinations of purified oFSH, hCG, rFSH, *r*LH and rPrl were carried out essentially by the method of Greenwood *et al.* (1963) except that it was done at 4°C, and β-mercaptoethylamine hydrochloride was used as a reducing agent. The specific activities of the radiolabeled oFSH and hCG were in the order of approximately 1–1·25 ($\times 10^5$) and 1·25–1·5 ($\times 10^5$) cpm/ng respectively. Typically 40 to 50% of the iodinated hormone was bound to excess receptor preparation (27000 g pellet obtained from freshly prepared testicular homogenate of 2 months old mature male rats).

Granulosa cell isolation

The animals were sacrificed by cervical dislocation. The ovaries quickly excised, freed of surrounding fat and connective tissues and placed in 2 ml Krebs-Ringer bicarbonate buffer containing 20 mM 4-(2 hydroxyethyl)-1-piperazine ethane sulphonic acid, 0·2% glucose, 0·1 % bovine serum albumin (pH 7·2)-Hepes-buffer. All handling of the tissues was done in a petri-dish containing Hepes-buffer and kept on crushed ice. The ovaries from each group of animals were pooled and the total ovarian wet weight was recorded in a torsion balance.

Granulosa cells were expressed into Hepes-buffer as described earlier (Vidyashankar and Moudgal, 1981). The volume of cell suspension of different treatment groups were adjusted on the basis of the ovarian weight so that an approximately equal number of cells per unit volume was obtained. Aliquots of cell suspension (100 μl) were used for *in vitro* incubation and DNA estimation. DNA was estimated by Burton's modified diphenylamine method (Giles and Myer, 1965) with the reagent volumes proportionately reduced to yield a 1-ml final volume. Calf thymus DNA was used as standard DNA preparation (sensitivity 5 μg/ml).

In vitro incubation and radioimmunoassay

The granulosa cells (100 μl aliquots) were incubated in triplicate with [^{125}I]-oFSH (2×10^5 cpm/tube) with or without a large excess of unlabelled oFSH in 3 ml glass tubes in a volume of 500 μl for 2 h at 37°C in a Dubnoff incubator at 60 oscillations per min. At the end of the incubation the tubes were centrifuged at 1500 *g* at 4°C, the cell pellets were washed with one ml cold Hepes-buffer and counted for bound radioactivity in a Packard Autogamma Counter. LH receptor concentration was monitored using [^{125}I]-hCG essentially by the method described for FSH receptor. Results are expressed as [^{125}I] -hormone bound/100 μg DNA. This assay has been validated with respect to hormone specificity and saturability. Graded amounts of oFSH (0·1-100 ng/tube) and pregnant mare serum gonadotropin (PMSG) (1 ng-1 μg/tube) competed with [^{125}I] -oFSH for FSH binding sites on the granulosa cells (50 μg calf thymus DNA equivalents) obtained from immature rats 72 h after a single injection of 4 mg DES in propylene glycol. oLH and hCG did not compete with [^{125}I] -oFSH at concentrations upto 5 μg/tube. The time course of [^{125}I] - oFSH binding to granulose cells showed a biphasic response with an initial rapid phase ofbinding in the first 15 min followed by a slower phase reaching maximal binding from 45–120 min of incubation. Non specific binding was less than 5 % of the total binding, and was of the order of 1% of labelled hormone input. [^{125}I] -oFSH binding to these rat granulosa cells was linearly related to cell concentrations when cell suspensions (in the range of 20-100 μg calf thymus DNA equivalents) were incubated with radiolabeled FSH (2×10^5 cpm/tube). Optimal cell concentration and labelled hormone were used in receptor binding studies to obtain saturation of receptors. Typically granulosa cells (50-60 μg calf thymus DNA equivalents) in each treatment group were used in radioreceptor assay.

Rat Prl, FSH and LH in the serum were determined by homologous radioimmunoassay using NIAMDD RIA kits. Progesterone and estradiol-17β in the ovarian tissue was estimated after homogenization in buffer and extraction with diethylether (X3) and reconstitution in 0·01M phosphate buffer, pH 7·4, containing 0·1 % gelatin. The free and bound labelled steroids were separated by dextran coated charcoal treatment. The sensitivity of the assay was 10 and 25 pg, the range 5–500 pg and 10–1000 pg per tube for estradiol and progesterone assays respectively.

In the following experiments, granulosa cells were obtained from pooled ovaries of rats of each treatment group, wherein the number of rats ranged from 4-8. Each experiment has been repeated more than twice and the results of a typical experiment have been indicated in all the cases.

Results

Induction of FSH receptors by DES; Time course and dose response

A single injection of 4mg of DES produced a 3 fold increase in FSH receptor concentration 72 h later (figure 1A). Thereafter a shallow drop in the receptor level was observed which was maintained until 144 h after the hormone injection. The increase in specific activity of FSH receptors ([^{125}I]-oFSH bound/100 μg DNA) paralleled the increase in ovarian weight and granulosa cell proliferation as measured by the increases in DNA, suggesting that an induction of FSH receptors had occurred following DES administration.

From the dose response of FSH receptor induction by different doses of DES, it was observed that maximal effect was obtained with 4mg dose, and consequently in all further studies this dose was used for induction of FSH receptors (figure IB).

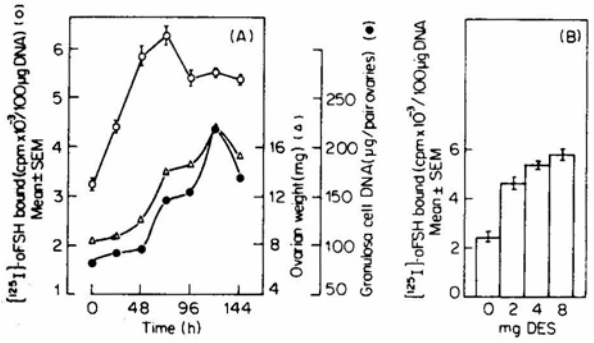

Figure 1. Time and dose-response curve illustrating the FSH receptor induction in granulosa cells following a single injection of DES. **A.** Indicates time course of receptor induction by 4mg DES. $P < 0{\cdot}002$ between 0 h and all other time points. **B.** Indicates dose response of receptor induction 72 h after injection of different doses of DES. $P < 0{\cdot}01$ between 0 and 4 or 8mg DES. FSH receptor concentration in the isolated cells was monitored as described under Materials and methods. Values are Mean ± SEM of triplicate determinations.

Ability of DES to induce FSH receptors in the presence of gonadotropin antisera

Since estrogens are known to effect pituitary gonadotropin release (Labrie *et al.,* 1978), it was essential to learn whether the FSH receptor induction by estrogens, could be a result of such an effect. The ability of DES to induce FSH receptors remained unchanged even when animals were treated with oFSH and oLH antisera to neutralize endogenous rat FSH and LH (table 1). These antisera in the amounts used were more than adequate to neutralize endogenous rat FSH and LH respectively.

Since in several earlier instances, investigators (Bergink *et al.,* 1973; Palmiter, 1975; Murty and Adiga, 1978) have observed that estrogen is capable of secondary stimulation of specific protein synthesis, it was considered worthwhile to see if a second injection of DES given 96 h after the first one does bring about secondary stimulation of the receptors. No such effect, however, was observed (table 2). Further, progesterone when given at a dose of 2 mg/rat was found not to inhibit the ability of DES to induce FSH receptors (table 3).

Table 1. Ability of DES to induce FSH receptors in the presence of gonadotropin antisera.

Treatment	CPM [^{125}I]-oFSH bound/100 μg DNA Mean ± SEM
Control	2167 ± 106
DES	4616 ± 137*
DES + oFSH a/s	5609 ± 205*
DES + oLH a/s	5548 ± 46*

Groups of rats received a single injection of 4 mg DES in 0·1 ml propylene glycol or vehicle on day 21. Monkey antiserum (300 μl) to oFSH and oLH was injected 2 h prior to DES injection. Controls received 300 μl of normal monkey serum. * $P < 0{\cdot}001$ compared to control value.

Table 2. Inability of DES to cause secondary stimulation of FSH receptor induction.

Injection of DES at (h)	Time of autopsy (h)	CPM [^{125}I]-oFSH bound/100 μg DNA Mean ± SEM
—	96	3125 ± 150
0	96	7733 ± 151
0 and 96	168	6425 ± 159
0	168	2750 ± 55

Groups of rats received either vehicle or 4 mg DES in 100 μl propylene glycol at the times indicated above. The animals were autopsied at the indicated time and FSH receptor concentration in isolated granulosa cells was monitored as described under Materials and methods.

Table 3. Effect of progesterone on the ability of DES to induce FSH receptors.

Treatment	CPM [^{125}I]-oFSH bound/100 μg DNA Mean ± SEM
Control	3250 ± 90*
Progesterone (2 mg)	3000 ± 105**
DES (4 mg)	6525 ± 190*
DES (4 mg) + progesterone (2 mg)	6575 ± 125**

Groups of rats received a single injection of vehicle (control), DES, progesterone, DES + progesterone. The animals were autopsied 72 h after the treatment and FSH receptor concentration in isolated granulosa cells was monitored as described under Materials and methods.
* $P < 0{\cdot}001$, ** $P < 0{\cdot}001$.

Characteristics of the induced receptor

Lineweaver-Burk plot of binding data obtained by incubating [^{125}I]-oFSH with granulosa cells from untreated and DES treated rats indicated no change in the affinity of binding of FSH to its receptor although a 2-fold increase in the concentration of the

receptors was observed in the cells from the DES treated group. Thus the newly induced FSH receptors appeared to be kinetically indistinguishable from the pre-existing ones (figure 2).

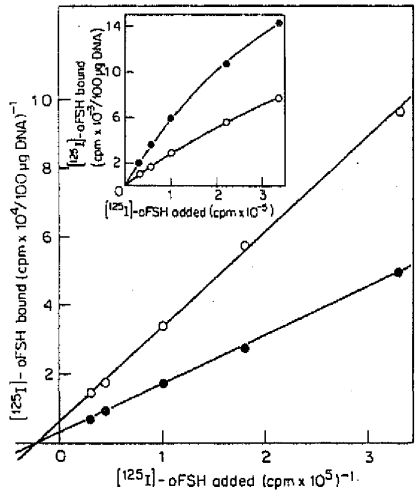

Figure 2. Lineweaver-Burk plot of binding data obtained by incubating [^{125}I]-oFSH with granulosa cells isolated from ovaries of untreated or 4 mg DES treated rats 72 h earlier. Binding was determined as described under Materials and methods. Linear regression was fitted by the least squares method. Half maximal binding was achieved in both cases at $4{\cdot}6 \times 10^5$ cpm. Binding sites (maximal cpm bound/100 μg DNA) calculated from the above, untreated 15,393 (○) and treated 35,983 (●).

The functionality of the induced receptors was assessed by determining the rate of [^{3}H]-leucine incorporation into proteins synthesized *in vitro,* in granulosa cells isolated from ovaries of groups of rats treated 72 h previously with DES or vehicle. While one set of controls and DES treated rats received 20 μg of oFSH (NIH FSH S15), 2 h prior to autopsy, another equivalent set of rats received saline only. It is evident that cells from DES treated rats respond to FSH significantly (figure 3) showing thereby that the induced receptors are truly functional.

Interaction of DES and FSH in inducing gonadotropin receptors in the immature rats

In view of our observation that estrogen induces FSH receptors in the absence of both endogenous LH and FSH and the earlier observations of Richards *et al.* (1976) that FSH induces its own receptors in hypophysectomized rats, the following experiments were undertaken to better understand the interaction between DES and FSH. In the first set, immature rats treated either with or without a single injection of DES, were injected 48 h later either saline or FSH (3 μg of highly purified oFSH, S 1528 C_2R) given every 12 h, the animals being autopsied 12 h after the last FSH injection. FSH treatment alone increased both FSH and LH receptors in the granulosa cells, whereas DES given alone, increased FSH receptors, but reduced the LH receptors when compared with the untreated controls (table 4). Injection of FSH to DES pretreated immature rats resulted in an apparent further increase in both FSH and LH receptor concentration.

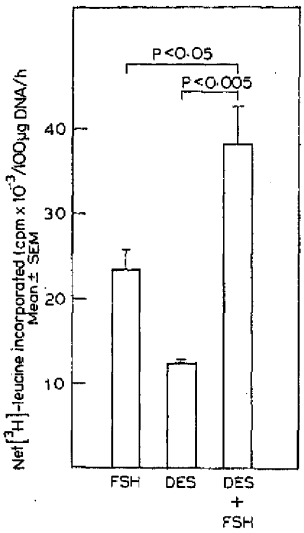

Figure 3. Demonstration of the functionality of induced receptors by DES: See text for experimental protocol. Granulosa cells isolated from the pooled ovaries of each group of rats were incubated in triplicate in glass tubes with 10 μCi of (L 4,5-^{3}H) leucine (105 Ci/m mol) in a final volume of 500 μl of Hepes-buffer, after oxygenation for 20 sec in a shaking incubator for 1 h at 37°C. The time course of [^{3}H]-leucine incorporation into cellular proteins was linear with time at 30, 60 and 90 min of incubation. At the end of the incubation, the tubes were spun, the cell pellets washed once with cold Hepes-buffer and resuspended in bufer and an equivalent volume of 20% trichloroacetic acid was added to precipitate the proteins. The precipitate was washed once with 10 % trichloroacetic acid, dissolved in 0·3 ml of formic acid, transferred to planchets, dried and counted in toluene PPO cocktail. An equivalent volume of cells was used for the estimation of its DNA content. The number of rats in the untreated group was 6 and the DES treated group was 4.

Table 4. Interaction of DES and FSH in inducing gonadotropin receptors.

Treatment	CPM [^{125}I]-oFSH bound/ 100 μg DNA Mean ± SEM	CPM [^{125}I]-hCG bound/ 100 μg DNA Mean ± SEM
(A) Control	3546 ± 141	1265 ± 4
(B) DES*	5689 ± 115	518 ± 140
(C) FSH*	6057 ± 413	2539 ± 142
(D) DES + FSH**	8186 ± 165	3453 ± 50

Groups of immature rats received the following treatment:
A: 100 μl of propylene glycol at 0 h followed by 100 μl of saline at 48, 60 and 72h.
B: 4 mg DES in 100 μl propylene glycol at 0 h and 100 μl of saline at 48, 60 and 72h.
C: Same as in group A except 3μg oFSH (S 1528 C_2R) in saline injected at 48, 60 and 72 h.
D: Same as in group B except 3 μg oFSH in saline injected at 48, 60 and 72 h.
All the animals were autopsied at 84 h and the FSH and LH receptor concentration in isolated granulosa cells monitored according to the procedure detailed under Materials and methods.
* $P < 0{\cdot}001$ compared with control.
* * $P < 0{\cdot}01$ D compared with group B and C.

However, analysis of data showed that this effect of DES and FSH in inducing FSH receptors was an additive effect. FSH was all the same more effective in inducing LH receptors in DES primed rats, when compared with untreated controls suggesting the involvement of estrogen in the LH receptor inductive process by FSH.

In the second set of experiments immature female rats received the first FSH (20 μg NIH oFSH SI5) injection along with DES at 0 h, the animals receiving additional FSH injections at 12 h, 24 h and 48 h. The animals were autopsied at 72 h, and the granulosa cells isolated monitored for FSH receptors. The results of this study also showed the effect of DES and FSH on receptor induction to be additive (table 5).

Role of prolactin in the FSH receptor induction by DES

The well established fact that estrogen brings about short term and long term release of prolactin from the pituitary (Meites *et al.,* 1972; Maurer and Gorski, 1977; Zyzek *et al.,* 1981) led us to investigate whether blocking prolactin secretion by EBC had any effect on the DES induction of FSH receptors. EBC (100 μg) inhibited the DES stimulated FSH receptor induction by 30 % (table 6). This inhibition was, however, totally relieved with ovine prolactin administration. Prolactin, when tried alone had no effect on the FSH receptor concentration.

Table 5. Interaction of DES and FSH in inducing FSH receptors.

Treatment	CPM [^{125}I]-oFSH bound/100 μg DNA Mean ± SEM
Control	7428 ± 216
FSH	11672 ± 695
DES	13498 ± 160
DES + FSH	17780 ± 763

See text, Results for experimental protocol. The granulosa cells were isolated and monitored for FSH receptor concentration as described under Materials and methods.

Table 6. Effect of blocking prolactin secretion by EBC on FSH receptor induction by DES.

Treatment	CPM [^{125}I]-oFSH bound/100 μg DNA Mean ± SEM
Control*	2667 ± 238
EBC	2517 ± 555
DES*†	6802 ± 59
DES + EBC**†	5082 ± 213
DES + EBC + oPrl**	7938 ± 60
DES + oPrl	7635 ± 264

Groups of rats received 4 mg DES or vehicle. 100 μg of EBC was injected 45 min prior to DES injection. oPrl 250 μg was administered every 12 h after the DES injection (total 3 mg/rat). FSH receptor concentration in isolated granulosa cells was monitored 72 h after DES injection as described under Materials and methods.

* $P < 0{\cdot}001$; ** $P < 0{\cdot}001$; † $P < 0{\cdot}002$.

Role of prolactin during cyclical follicular maturation

Administration of EBC to cycling rats at noon of proestrus significantly inhibited FSH (~ 30 %) and LH (~ 45 %) receptor levels (figure 4) in the granulosa cells of the ovaries isolated on diestrus II. This was suggestive of proestrus prolactin surge having a role on the overall follicular maturation process. Administration of EBC on proestrus noon had no effect on the surge of FSH and LH occurring in the evening of the same day but markedly reduced, as expected, Prl levels (table 7). To better understand the role of Prl surge in follicular maturation, rats injected with EBC at noon of proestrus were autopsied on diestrus II and ovarian estradiol- 17 β, and progesterone levels estimated. Another group of rats received EBC at noon on proestrus followed by ovine Prl at 1700 h the same day to simulate the proestrus Prl peak. Compared to the untreated control a drastic decrease (93 %) in the ovarian tissue estradiol-17 β occurred following EBC injection on proestrus. However, the luteal function was not affected by Prl lack as evidenced by no change in the tissue progesterone levels of control and EBC treated rats. Inhibition could be totally relieved by supplementing EBC treated rats with

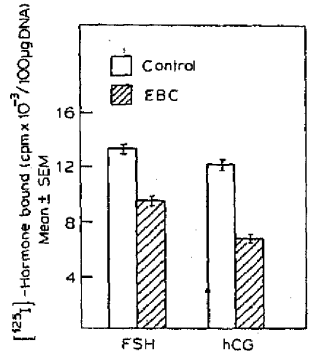

Figure 4. Role of Prl during follicular maturation process in adult cycling rats; Animals (n = 3) were given 100/μg EBC or vehicle at 1200h on proestrus and autopsied at 1400h on diestrus II (3 days later). The granulosa cells obtained from pooled ovaries of each group were monitored for FSH and LH receptor concentration as described under Materials and methods. $P < 0{\cdot}01$ and $P < 0{\cdot}002$ between control and EBC treated groups for FSH and LH receptor concentrations respectively.

Table 7. Effect of administration of EBC on the proestrus FSH, LH and prolactin levels in the serum.

Treatment	ng rFSH RP_1/ml Mean ± SEM	ng rLH RP_2/ml Mean ± SEM	ng rPrl RP_3/ml Mean ± SEM
Control	1070 ± 162	26·92 ± 1·68	111 ± 15*
EBC	816 ± 16·6	27·2 ± 4·3	16·6 ± 1·1*

EBC (100 μg in 50% ethanol) or vehicle alone was administered to adult cycling rats on proestrus noon, animals were autopsied at 1700 h of the same day, abdominal vein blood was collected and serum separated. The FSH, LH and prolactin concentration in the serum were estimated by specific radioimmunoassay using NIAMDD RIA kits, n in each group was 3.
* $P < 0{\cdot}005$.

exogenous Prl on the evening of proestrus (table 8). The higher tissue progesterone seen in Prl treated group is perhaps a reflection of its luteotropic activity.

In yet another experiment following a protocol described in legend to table 8, granulosa cells isolated from untreated control, EBC and EBC + oPRL treated groups were incubated with testosterone and with or without FSH. The ability of granulosa cells to aromatize testosterone to estradiol in the presence of FSH was reduced in the EBC treated group, supplementing such rats with oPrl once again appeared to restore this activity (table 9).

Table 8. Effect of EBC administration at noon of proestrus on the ovarian progesterone and estradiol content measured on diestrus II.

Group	Tissue estradiol pg/100 μg DNA Mean ± SEM	Tissue progesterone ng/100 μg DNA Mean ± SEM
I Control	1373 ± 247*	72·35 ± 11·29
II EBC	59 ± 28*	71·6 ± 9·02
III EBC + oPrl	1259 ± 135	151·63 ± 11·33

The first group of adult cycling rats received 50 % ethanol (100 μl) at noon and saline (100 μl) at 1700h on the day of proestrus. The second group received EBC (100 μl in 100 μl 50% ethanol) at noon of proestrus. The third group received EBC (100 μg in 100 μl 50 % ethanol) at noon plus 1 mg of oPrl at 1700 h of proestrus. Animals were autopsied 3 days later, (diestrus II) the ovaries excised and stored frozen in saline until assayed for their contents of estradiol and progesterone. Three animals were used in each group.
* $P < 0{\cdot}005$.

Table 9. Effect of administration of EBC at noon of proestrus on the ability of granulosa cells isolated on diestrus II, to convert testosterone to estradiol17β.

	pg estradiol/100 μg DNA		
		Testosterone 3×10^{-7} M	
Treatment	No addition	−FSH	+FSH
Control	1612 ± 95	5626 ± 315	8820 ± 95*
EBC	971 ± 32	3265 ± 73	2887 ± 150*
EBC + oPrl	1378 ± 61	4859 ± 155	7997 ± 470

The protocol of the experiment is detailed in the legend to table 8. The granulosa cells isolated from pooled ovaries of control, EBC and EBC + oPrl treated groups were incubated for 4 h at 37°C with testosterone and with or without oFSH in a total incubation volume of 500 μl. The duration of incubation and the testosterone concentration was chosen according to Vidyashankar and Moudgal (1981). The estradiol secreted into the medium was assayed by an RIA described under Materials and methods. Values are Mean ± SEM of triplicate determinations. Three animals were used in each group.
* $P < 0{\cdot}001$.

Discussion

From the foregoing it is evident that DES, an estrogen, is endowed with an intrinsic ability to induce FSH receptors in the immature intact rat. Estrogens have been shown to increase granulosa cell proliferation and growth of a large number of preantral

follicles in the immature hypophysectomized female rat and prolonged treatment with DES is shown to increase ovarian uptake of [^{3}H]-thymidine and [^{3}H]-FSH *in vivo* (Goldenberg *et al.,* 1972). In the hypophysectomized immature rats estradiol alone appears to have no effect on the number of FSH receptors when expressed as cpm of [^{125}I] -FSH bound per μg of DNA of isolated granulosa cells but it does seem to enhance the ability of FSH to increase its own receptors (Louvet and Vaitukaitis, 1976; Ireland and Richards, 1978).

In contrast to the above we find that in the intact immature rat a single injection of DES, in addition to effecting the growth of follicles resulting in granulosa cell proliferation, does bring about a concomitant increase in the specific activity of the FSH receptors expressed here as [^{125}I] -FSH bound per unit DNA. The kinetic property of the induced receptors itself did not change indicating that induction of FSH receptor had truely occurred. Evidence for the functionality of the induced receptors is also provided by demonstrating that granulosa cells from DES treated rats incorporate significantly higher amount of [^{3}H] -leucine into protein compared to appropriate controls. The observation that even in the absence of endogenous gonadotropins, DES does bring about an increment in receptor concentration, underlines the specificity of the effect. We are presently unable to offer any explanation for this difference in the conclusion of earlier workers and ours excepting to point out that it could stem from the model system and repeated high dosage of estrogen used by them. The results of the experiments wherein both DES and FSH were given together, actually is suggestive of each having an independent inductive effect on granulosa cell FSH receptor levels, administering them together producing an additive effect. This is in contrast to the clear stimulatory effect FSH exhibits with respect to LH receptor induction when given to DES treated rats. Earlier workers have shown that FSH increases the proliferation rate in granulosa cells (Nakono *et al.,* 1975) and more so in combination with estradiol (Rao *et al.,* 1978). Both estrogens and FSH are believed to be essential for the development of LH receptors of rat granulosa cells (Zeleznik *et al.,* 1974; Richards *et al.,* 1976; Nimrod *et al.,* 1977; Sheela Rani *et al.,* 1981). Richards *et al.* (1976) also observed a decrease in LH receptor upon estradiol administration to hypophysectomized immature rats and found an enhanced stimulation of LH receptors in estradiol-hFSH treated rats. We essentially confirm the above results.

The present study has uncovered the possible role of prolactin in inducing/maintaining gonadotropin receptors. Thus the administraion of EBC to immature rats prior to DES treatment, significantly blocked the FSH receptor induction by DES, and the inhibition was promptly relieved by prolactin administration. The observation that prolactin alone at the doses tried had no effect in inducing FSH receptor, suggest it having only a permissive role in the FSH receptor inductive process by estrogens.

Extending this line of investigation to cycling rats we have observed that the prolactin surge which appears along with LH and FSH surges on the evening of proestrus could be having an effect on follicular maturation. Blockade of prolactin surge significantly reduced gonadotropin receptors in the granulosa cells isolated from the ovary on diestrus II (*i.e.*, 3 days later). EBC at the dose tried seemed to reduce only the Prl levels, the LH and FSH levels showing no discernable change. This treatment, however, resulted in a drastic reduction in the ovarian tissue estradiol levels on diestrus

II, and that this was a specific effect of Prl lack was shown by the restoration of the estradiol level by exogenous administration of Prl on the evening of proestrus. The ability of granulosa cells isolated from EBC treated rats to aromatize testosterone to estradiol, in response to FSH *in vitro* was also reduced, but this could once again be restored by administering EBC treated rats Prl on the proestrus evening to simulate a Prl surge. These results would thus suggest that prolactin surge could be having a role in the follicular maturation process and perhaps this is being achieved by modulating in some way the effect of FSH on granulosa cells.

Granulosa cells have been shown to possess Prl receptors on their cell surface. The Prl receptors are few in the granulosa cells of immature rat ovary, but as the follicles develop from the preantral to the large antral stage, these receptors apparently increase. Induction of Prl receptors *in vivo* by FSH treatment to estrogenized immature hypophysectomized rats has been demonstrated (Richards and Williams, 1976; Wang *et al.,* 1979). FSH could also stimulate induction of prolactin receptors in cultures of granulosa cells from DES treated immature hypophysectomized rats (Wang *et al.,* 1979). However, Prl has been shown to inhibit FSH stimulated aromatase in cultures of granulosa cells, obtained from DES primed rats (Dorrington and Gore-Langton, 1982). McNatty (1979) has observed that high levels of Prl in the presence of normal plasma FSH levels has antigonadal effects in woman. In the lactating rat, administration of oPrl effectively reduced the increase in nonluteal estradiol following PMSG treatment (Maneckjee *et al*., 1977). Progesterone synthesis by human granulosa cells *in vitro* is suppressed by high concentrations of Prl (McNatty *et al*., 1974). Prl has been shown to inhibit basal and gonadotropin stimulated secretion of progesterone and estradiol in human ovaries (Demura *et al.,* 1982). It has been concluded that Prl acts directly on the rat granulosa cells to inhibit the induction of the aromatase activity by FSH, resulting in a decrease in the amount of estrogen synthesized (Dorrington *et al*., 1983).

The above mentioned observations made in hyperprolactonemic conditions and in *in vitro* cell culture studies are in contrast to the results presented in this study wherein endogenous Prl secretion blocked by EBC reduced receptor concentration while supplementation with exogenous Prl prevented reduction of total ovarian estrogen levels and the aromatase activity, brought about by EBC. Using FSH-primed LH-treated immature rats, Holt and Richards (1975) observed reduced [^{125}I]-hCG binding to membrane fractions and serum progesterone following ergocryptine administration. Simultaneous treatment with ergocryptine and Prl completely reversed the effects of ergocryptine. Since Prl secretion was blocked with EBC, a drug having a dopaminergic effect, one should view with caution some of the conclusions, as EBC in addition to reducing Prl levels could also have effects at other loci.

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