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Blockade of Estrogen Synthesis with an Aromatase Inhibitor Affects Luteal Function of the Pseudopregnant Rat

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The luteotropic action of estrogen (E) was investigated using immature pseudopregnant rat as the model and CGS 16949A (Fadrozole hydrochloride), a potent aromatase inhibitor (AI), to block E synthesis. Aromatase activity could be inhibited by administering CGS 16949A (50 μg/day/rat) via a mini osmotic Alzet pump (model 2002) for 3 days during pseudopregnancy. This resulted in significant reduction of serum (40%, $P < 0.05$) and intraovarian (70.6%, $P < 0.001$) estradiol-17β (E_2) levels. The serum and intraovarian progesterone (P_4) levels as analyzed on day 4 of pseudopregnancy were also reduced by ⩾ 50% (for both, $P < 0.01$). Simultaneous administration of estradiol-3-benzoate (E_2B) via an Alzet pump during the AI treatment period at a dose of 1 μg/day could completely reverse the AI induced reduction in P_4 secretion. The luteal cells of experimental rats depleted of E *in vivo* showed a significantly reduced response upon incubation with hCG or dbcAMP *in vitro* ($P < 0.05$ and 0.001, respectively). Addition of E_2 (500 pg/tube) at the time of *in vitro* incubation was able to partially increase the responsiveness to hCG. The luteal cell LH/hCG receptor content and the affinity of hCG binding to the receptor remained unchanged following AI treatment *in vivo*. Both esterified and total cholesterol content of luteal cells of rats treated with AI *in vivo* was significantly high ($P < 0.05$) suggesting that E lack results in an impairment in cholesterol utilization for steroidogenesis. The results clearly show that E regulates luteal function in the pseudopregnant rat by acting at a non-cAMP mediated event and this perhaps involves facilitation of cholesterol utilization at the mitochondrial level for P_4 synthesis.

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INTRODUCTION

Earlier investigations have shown that in the pregnant rat luteinizing hormone (LH) stimulates luteal synthesis of testosterone (T) which in turn is converted to estradiol-17β (E_2) by the highly active aromatase system present in the corpus luteum [1–3]. The locally formed E_2 presumably stimulates progesterone (P_4) synthesis [4, 5] by biochemical mechanisms yet to be clearly understood. In the pregnant rat corpus luteum, it is presumed that E_2 stimulates P_4 synthesis by regulating lipoprotein receptor content, an event which modulates the cellular uptake of lipoprotein substrates [6, 7]. In addition, E_2 treatment seems to bring about hypertrophy and vascularization of the corpus luteum as well as enhancement in the synthesis of specific proteins like that of sterol carrier protein-2 [7, 8]. Most of the studies carried out hitherto to establish a luteotropic function for E_2 have used the hypophysectomized, hysterectomized pregnant rat as the animal model. Some of the conclusions arrived at using this model could be arbitrary as multiple surgeries used could interfere with the synergistic action of hormonal factors other than estrogen (E) in the overall regulation of luteal function. The availability of a new generation of aromatase inhibitors which specifically inhibit the conversion of androgen to E [9, 10] has enabled us to devise a suitable animal model for the study of the role of E in reproductive functions in the female mammal [11, 12]. In the present communication the luteotropic action of E is examined using pseudopregnant rat as the animal model and Fadrozole hydrochloride (CGS 16949A) as the aromatase inhibitor (AI) to specifically block E synthesis.

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MATERIALS AND METHODS

Hormones and chemicals

PMSG and hCG (CR 127) were from NICHD, Bethesda, U.S.A. (courtesy of Dr G. Bialy). Tritiated progesterone (88 Ci/mmol) and estradiol (90 Ci /mmol) were purchased from Amersham, U.K. Aromatase inhibitor CGS 16949A (Fadrozole hydrochloride) was a product of Ciba-Geigy, Basel, Switzerland [10]. Mini osmotic pumps (model 2002) were procured from Alza Corp., Palo Alto, CA, U.S.A. Dulbecco's Modified Eagle's Medium (DMEM) was from GIBCO, U.S.A. Hepes, dbcAMP and all other general chemicals were obtained from Sigma Chemical Co., MO, U.S.A.

Animals and treatment schedule

Immature female Wistar rats (21–23-day old) bred in the Central Animal Facility of the Institute were kept in groups of 4–5 rats per cage in a well ventilated room having a light:dark regimen of 14:10 h. The animals were fed a standard pelleted diet (Lipton, India) and water *ad libitum*.

All the animals received 30 IU of PMSG on day 0 of treatment followed 48 h later by 50 IU of hCG by subcutaneous route. An Alzet mini osmotic pump containing the AI, CGS 16949A (release rate of 50 μg/day), was implanted subcutaneously in the experimental animals 24 h after hCG administration. The pump was maintained until autopsy on the 6th day after PMSG administration (4th day of pseudopregnancy). Two additional groups of rats received, along with AI, estradiol-3-benzoate (E_2B) at a dose of 0.1 and 1.0 μg/day. Both AI and E_2B were delivered via the mini pump.

Collection of blood samples and autopsy

Blood was collected from control and experimental animals under ether anaesthesia by tapping the abdominal vein, just before autopsy. The animals were then killed by an overdose of anaesthesia, the ovaries and the uteri carefully dissected from the adhering tissue and quickly weighed.

Radioimmunoassays

E_2 and P_4 assays of sera samples were carried out as described earlier [13, 14]. The ether extracted samples were suspended in a known volume of gelatine phosphate buffered saline (GPBS) and was used for steroid determination. Intra ovarian E_2 and P_4 were quantified by RIA after ether extraction of the homogenates. The *in vitro* luteal cell incubates were used directly without extraction for the assay. The P_4 antiserum used was a kind gift from Dr Joshi, IRR Bombay, the characteristics of which have been provided earlier [13, 14]. The E_2 antiserum used was raised in our laboratory and this cross-reacted with estrone and estriol to the extent of 3.7 and 1.1%, respectively, and with P_4 and T to < 0.1 %.

Luteal cell in vitro bioassay

The ovaries having corpora lutea from each of the experimental animals were rapidly removed, pooled together and placed in the cold DMEM medium containing 25 mM Hepes, 0.37% $NaHCO_3$ and 0.1% BSA (pH 7.4). The ovarian luteal cells were isolated essentially according to the method described earlier [15]. Briefly, the ovaries from each of the experimental groups (4 pairs) containing corpora lutea were cut into small pieces, suspended in 3 ml of DMEM containing 2% BSA and 0.2% collagenase and incubated at 37°C in a shaking water bath for 15 min. To this was added 2 ml of DMEM containing 2% BSA and single cells were obtained by repeatedly aspirating and releasing the suspension with a pipette for about 1 min.

After the debris was allowed to settle, the supernatant containing cells was aspirated. The debris was subjected to collagenase digestion twice more, the supernatant from different digests pooled and the cells collected by centrifugation washed thrice to remove excess collagenase. The final cell pellet was suspended in a fixed volume of the medium.

The luteal cells in the DMEM medium were preincubated at 37°C for 2 h in a shaking water bath followed by washing and were suspended in a known volume of medium. The cells were counted in a haemocytometer; 10^5 viable cells per tube (as determined by trypan blue dye exclusion test) were incubated in triplicate in the presence of increasing concentrations of hCG (50 pg, 500 pg, 5 ng, 50 ng) or dbcAMP (62.5, 125, 250, 500 μM) with or without E_2 (500 pg/tube) in a total volume of 0.5 ml (in 22 × 75 mm glass tubes) for 3 h at 37°C in a shaking water bath at 60 oscillations per min. In a separate experiment 10^5 viable luteal cells from control animals were also incubated with 10^{-6}M CGS 16949A with (50 ng) or without hCG in triplicate as above. After incubation the tubes were kept frozen at −20°C until assayed for P_4.

Luteal tissue preparation for LH (hCG) receptor assay

Iodination of hCG was carried out according to the method of Fraker and Speck [16]. The specific activity of the iodinated hormone ranged between 2×10^7 to 3×10^7 cpm/ μg protein.

LH (hCG) receptor content was measured by radio receptor analysis as detailed earlier [17]. Initially the B_{max} for the binding of [^{125}I]hCG to the luteal cell membrane was determined by incubating varying amounts of the membrane preparation (5–150 μl of 60 mg/ml preparation) with [^{125}I]hCG alone (200,000 cpm) or with 1000-fold excess of unlabelled hCG at 37°C for 1 h. The total assay volume was made up to 600 μl with the buffer containing 0.1% BSA [18]. The reaction was terminated by dilution with the cold buffer and centrifuged at 4000 rpm for 20 min at 4°C. The pellets were resuspended and washed. The radioactivity of the washed pellets was

determined in an automatic gamma counter. The difference between total binding and that observed in the presence of excess unlabeled hCG provided specific binding. Similarly a [^{125}I]hCG saturation binding analysis was carried out by incubating the aliquots of the membrane fractions (corresponding to 50% of the B_{max}) with increasing concentrations of [^{125}I]hCG with or without unlabeled hCG and specific binding was obtained as described above. The Scatchard plot analysis of these values gave the capacity and the affinity of the LH (hCG) receptors that can bind to hCG. All the binding data have been expressed as ng of hCG bound per 100 μg of protein present in the membrane receptor.

Estimation of luteal cholesterol and cholesterol ester

The methods used for the extraction of free and esterified cholesterol were essentially similar to those described by Major *et al.* [19] and Pokel *et al.* [20]. Briefly, known amount of corpora lutea were homogenized in 2 ml of cold (0–4°C) saline. The contents were washed into 30 ml conical glass centrifuge tubes, by repeatedly rinsing the homogenizer with chloroform–methanol mixture (2:1) and the total volume was made up to 20 ml. At this stage, trace amounts of [^{3}H]P_4, approx. 10,000 cpm were added to correct for procedural losses of the steroids during extraction. After allowing the tubes to stand at room temperature for 1 h, 4 ml of saline was added to each tube, and after brief shaking they were centrifuged. The chloroform layer in each tube was transferred to separate small round bottomed flasks and the solvent was evaporated by blowing N_2 through a manifold.

Free cholesterol was estimated according to the method of Glick *et al.* [21]. In brief, the sample residues of each flask were dissolved in 1 ml of absolute ethanol. Standard cholesterol (10–100 μl) or samples (20–200 μl) were made up in 1 ml of absolute ethanol. To each tube 1 ml of the diluted reagent was added and slowly mixed. The colour developed was read after 1 h at 558 nm. Tubes containing only absolute ethanol and equal volume of reagent served as blanks.

Total cholesterol was estimated as per the procedure of Abell *et al.* [22]. Briefly, the extracted samples were treated with alcoholic KoH to saponify the cholesterol esters, the cholesterol was then extracted into a known volume of petroleum ether after dilution of alcoholic solution with water. The cholesterol in an aliquot of the reagent was measured by using Liberman–Burchard reagent.

Statistics

Each treatment group (of all individual experiments) was made up of a minimum of 4 animals and each experiment was repeated in its entirety at least thrice to ensure obtaining reproducible results. Significant difference between groups was evaluated either by Student's t-test or ANOVA test depending on whether the comparable groups were 2 or more.

RESULTS

Effect of administering aromatase inhibitor on E_2 and P_4 levels in serum and ovary

Inhibition of aromatase activity *in vivo* during pseudopregnancy for 3 days resulted in 40 ($P < 0.05$) and 71 ($P < 0.001$) percent reduction in serum and intraovarian E_2 levels, respectively. This was accompanied by a $\geq 50\%$ reduction in serum ($P < 0.01$) and intraovarian ($P < 0.01$) P_4 levels (Table 1). Though the ovarian weight of AI treated animals showed no change over controls, the ovaries looked distinctly pale in colour compared to the controls suggesting an impairment in blood supply.

Supplementation with exogenous E_2B at a dose of 1.0 μg, but not 0.1 μg, per day, could reverse the AI induced reduction in serum P_4 level (Table 2). The reduction in uterine weight seen following AI treatment, however, could be reversed with either of the supplemental E_2B doses (Table 2).

Responsiveness of the luteal cells of pseudopregnant rats treated with AI in vivo to added hCG or dbcAMP in vitro

Following blockade of endogenous E synthesis by AI treatment *in vivo*, progesterone production by the

Table 1. Effect of blocking estrogen synthesis during luteal phase with an AI on steroid hormone profile in pseudopregnant rats. 21–23-day-old female rats were induced to become pseudopregnant by giving an ovulatory dose of hCG (50 IU/rat) 48 h after an injection of 30 IU of PMSG. Each of the experimental animals were implanted 24 h after hCG administration an Alzet miniosmotic pump releasing AI at 50 µg/day. The control and experimental animals were killed on the 4th day of pseudopregnancy, serum was collected, reproductive organs were weighed and the serum and the intraovarian steroid contents assayed

Treatment groups	Wt. of the ovary (mg) (Mean ± SE)	Wt. of the uterus (mg) (Mean ± SE)	Serum E_2 (pg/ml) (Mean ± SE)	Serum P_4 (ng/ml) (Mean ± SE)	Intraovarian E_2 (pg/paired ovary) (Mean ± SE)	Intraovarian P_4 (ng/paired ovary) (Mean ± SE)
Control ($n = 9$)	106.7 ± 4.5	102.9 ± 3.4	209.0 ± 27.4	72.5 ± 9.1	356.8 ± 56.0	238.8 ± 42.1
AI treated ($n = 9$)	95.3 ± 4.7	60.8 ± 2.4***	127.3 ± 13.5*	36.5 ± 6.7**	104.6 ± 10.2***	102.5 ± 17.7**

*$P < 0.05$.
**$P < 0.01$.
***$P < 0.001$.

Table 2. Demonstration of the ability of estrogen supplemented in vivo to override the inhibitory effect of AI on luteal progesterone secretion in pseudopregnant rats

Group	Treatment	Wt. of uterus mg (Mean ± SE)	Serum progesterone mg (Mean ± SE)
A	Control ($n = 12$)	94.8 ± 3.5	63.6 ± 7.1
B	AI ($n = 12$)	63.7 ± 2.4*	33.8 ± 6.5†
C	AI + E_2 ($n = 3$)	81.0 ± 13.8	32.7 ± 1.2†
D	AI + E_2 ($n = 4$)	92.0 ± 6.4	69.7 ± 11.1

Experimental protocol essentially similar to that in Table 1. Animals in groups C and D received 0.1 and 1.0 μg E_2B/day/rat respectively on days 1–3 of pseudopregnancy. All animals were autopsied on day 4 pseudopregnancy. *n* of each group is indicated in parentheses. Results analysed for statistical significance by ANOVA test.

*Uterus weight significantly different from A and D ($P < 0.001$)

†Serum progesterone significantly different from A and D ($P < 0.02$)

isolated luteal cells *in vitro* was significantly (from <0.05 to <0.001) inhibited (Figs 1 and 2) in response to added hCG (from 0.05 to 50 ng/tube) or dbcAMP (from 62.5 to 500 μM). The inhibition in response to added hCG, however, could be reversed marginally by co-incubating with 500 pg E_2 per tube (Fig. 1).

Addition of AI (10^{-6} M/tube) *in vitro* to luteal cells of control animals did not result in significant alteration in the *in vitro* production of basal (control: 12.3 ± 0.7 vs treated: 10.2 ± 0.9 ng P_4/3h/10^5 cells/500 μl) as well as hCG induced (50 ng/tube) P_4-secretion (control: 52 ± 3.1 ng vs treated: 43.3 ± 5.7 ng P_4/3h/10^5 cells/500 μl).

Effect of administration of aromatase inhibitor in vivo on luteal LH (hCG) receptor concentration and cholesterol levels

The [^{125}I]hCG binding assay showed that reduction in intraluteal E did not result in any change in either the LH (hCG) receptor content or the affinity of hCG binding to the receptor (Table 3).

The esterified as well as total cholesterol level of luteal cells of the AI treated animals, however, was observed to be significantly high (Table 4) compared to the controls ($P < 0.05$ and $P < 0.01$, respectively).

DISCUSSION

The pseudopregnant rat model system has been used extensively in studying the influence of various factors on luteal function [23–29]. Estrogen synthesized in the ovary either by the follicles, as in the case of the rabbit, or locally in the corpus luteum, as in the case of the primate and rat, has been implicated in regulating luteal function. In the rat E is presumed to act along with prolactin in supporting luteal P_4 secretion during specific times of pregnancy. Both E and prolactin do not bring about a dose-dependent increase in P_4 synthesis. Prolactin is known to specifically promote P_4 secretion by inhibiting its conversion into 20α-OH progesterone, a biologically inactive metabolite. Gibori and coworkers [4, 7, 25] used a hypophysectomized and hysterectomized pregnant rat model to understand better the role of T and E in regulating P_4 secretion. Hypophysectomy [6, 25] or specific LH deprivation [5] of the pregnant rat is known to result in a drastic reduction in both E and P_4 concentrations. On supplementation of such animals with a pharmacological dose of E or T *in vivo*, the ability of the luteal cells to synthesize P_4 was largely restored.

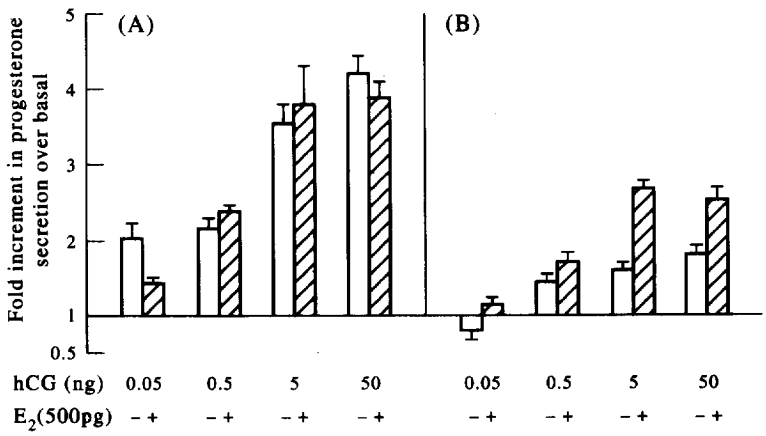

Fig. 1. *In vitro* responsiveness of the luteal cells from the control (A) and AI treated (B) pseudopregnant rats to hCG in the presence or absence of E_2. Collagenase digested luteal cells (10^5) suspended in 500 μl of DMEM were incubated with increasing concentrations of hCG with or without E_2 (500 pg) for 3 h. The experiment was carried out thrice with 3–4 animals in each treatment group. The pooled cells from each treatment group were incubated with the hormones in triplicate and the secreted P_4 from each tube was assayed in duplicate. The values are expressed as the fold increment in P_4 secretion over the basal and are the mean ± SE of a representative experiment. (Basal P_4 secretion control: 12.3 ± 0.7/10^5 cells/3h/500 μl, treated: 9.9 ± 0.5 ng/10^5 cells/3h/500 μl).

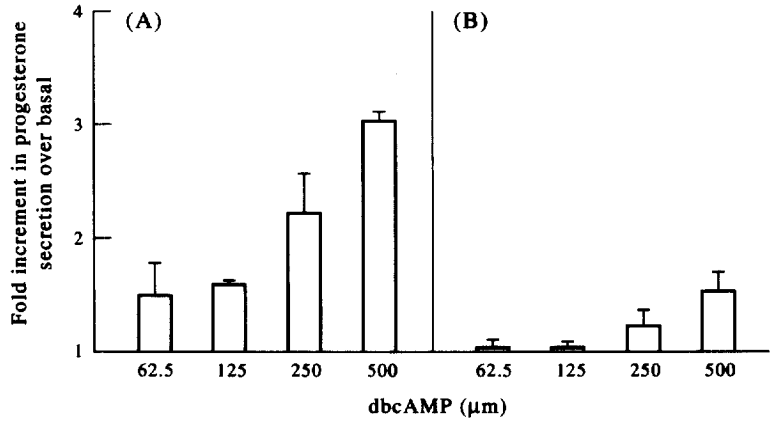

Fig. 2. *In vitro* responsiveness of the luteal cells from the control (A) and AI treated (B) pseudopregnant rats to dbcAMP. 10^5 luteal cells suspended in 500 μl of DMEM were incubated with increasing concentrations of dbcAMP for 3 h. The experiment was carried out thrice with 3–4 animals in each treatment group. The pooled cells from each treatment group were incubated with dbcAMP in triplicate and the secreted P_4 from each tube was assayed in duplicate. The values are expressed as the fold increment in P_4 secretion over the basal and are the mean ± SE of a representative experiment. For basal secretion rate refer to Fig. 1 legend.

In the current investigation using intact pseudopregnant rats, a significant reduction in E_2 levels has been achieved by administering micro doses of a highly specific aromatase inhibitor. The ability of this AI to specifically block E but not the synthesis of other steroid hormones has been shown earlier [9, 10]. Granulosa cells from ovaries of PMSG primed rats [11] as well as hFSH stimulated monkeys [12] treated with AI *in vivo*, have been observed to continue respond normally to hCG/FSH *in vitro*. Besides this, the cycling monkey treated with AI during the mid-luteal phase responds to hCG by producing a normal quantum of progesterone [30]. These studies essentially show that the AI used here has no effect *per se* on steroidogenic enzymes not involved in aromatization. The observation that AI added *in vitro* was unable to alter the basal or hCG induced P_4 secretion by the luteal cells of control animals during short term incubation studies suggests that (a) to determine the role of E in luteal function it is necessary to block E support for a continuous and longer period as done in *in vivo* studies and (b) AI as shown by the *in vitro* studies with luteal cells of control rats does not by itself have any nonspecific effect on luteal function. The dose of AI added here (10^{-6} M) has been shown, based on an earlier independent study with monkey luteal cells, to totally block E synthesis [30]. The reversal of the AI induced reduction in P_4 secretion in pseudopregnant rats (present study) by exogenously supplemented E_2B (1 μg/day) clearly demonstrates that the AI, CGS 16949A impairs P_4 production specifically by blocking E synthesis. Such blockade of E synthesis *in vivo* not only leads to a significant reduction in serum P_4 levels but also to a block in the ability of luteal cells to respond to added hCG *in vitro*. Addition of 500 pg of E_2 per tube during incubation appeared to enhance the responsiveness of luteal cells to relatively higher concentrations of hCG (5 and 50 μg/tube) but the reversal was not complete. Tekpetey and Armstrong [26, 27] have reported that while E_2 marginally inhibited the *in vitro* production of P_4 by cultured rat luteal cells catechol estrogen in a dose range of 2–10 ng/ml stimulated P_4 production.

The current observation that the luteal LH receptor concentration as well as its binding affinity of hCG remained unchanged following marked reduction in E

Table 3. Affinity and capacity of ovarian LH receptors of pseudopregnant rats treated with aromatase inhibitor. The membrane receptors prepared from the luteinized ovarian tissue of the AI treated and control pseudopregnant animals were analysed for the affinity and capacity of the LH receptors. The experiment was done 4 times with 3–4 animals in each treatment group. The values are the mean of the duplicate assay carried out from the samples of a representative experiment

Experimental groups	K_a	Capacity (ng hCG/100 μg protein)
Control	$0.13 \times 10^9\ M^{-1}$	9.3
AI treated	$0.15 \times 10^9\ M^{-1}$	8.4

Table 4. Effect of blocking estrogen synthesis with an aromatase inhibitor on free and esterified cholesterol level of luteal tissue of pseudopregnant rats. The corpora lutea (CL) from the control and AI treated pseudopregnant rats were homogenized and the free and total cholesterol content was estimated in the extracted samples. The values are the mean ± SE of 3 experiments of 3–4 animals in each treatment group

	Free cholesterol (μg/mg CL)	Esterified cholesterol (μg/mg CL)	Total cholesterol (μg/mg CL)
Control	5.3 ± 0.7	6.1 ± 0.6	11.4 ± 0.3
AI treated	6.4 ± 0.6	12.2 ± 1.7*	18.6 ± 1.4**

*$P < 0.05$.
**$P < 0.01$.

availability *in vivo* indicated that E is not affecting luteal function by regulating LH receptor induction/turnover. The fact that the luteal cells of such animals showed a significantly reduced response to dbcAMP added *in vitro* suggested that lack of E could be affecting a non-cAMP dependent step in steroidogenesis.

Estrogen presumably regulates luteal function of the pregnant rat by increasing the availability of cholesterol substrate for P_4 synthesis by stimulating cholesterol uptake, intra-cellular transport and synthesis [6, 31]. Treatment of pregnant rats with E_2 has been observed to result in marked enhancement of luteal content of sterol carrier protein (SCP2), a cholesterol transport protein [8]. Such treatment, however, had no effect on either the mRNA or protein content of the cholesterol side chain cleavage (scc) enzyme. Gibori *et al.* [7] claim that E_2 regulates P_4 production by increasing the availability of mitochondrial cholesterol by stimulating cholesterol synthesis from its esters by upregulating the high density lipoprotein (HDL) receptor in the luteal cells [2]. The current study clearly indicates that a significant depletion in E levels *in vivo* has no effect on the total intracellular cholesterol pool *per se*. The significant increase in cholesterol ester concentration seen, however, would suggest that this is occurring as a consequence of blockade in its transport to mitochondria and utilization for P_4 synthesis. Treatment of pseudopregnant rats with LH antibody has been shown to result in significant reduction in cholesteryl esterase and a marked enhancement in ester synthetase activity leading to an accumulation of luteal cholesterol ester levels [32]. Since blockade of LH action also results in reduction in estrogen synthesis in the rat it is possible that E promotes luteal P_4 production by regulating cholesteryl esterase activity. It appears from the above that in the rat LH regulates luteal progesterone production by stimulating the synthesis of estrogen and cAMP, each one of these in turn acting as secondary stimulators of steroidogenesis albeit at different steps in the pathway. It may be of interest here to draw attention to the fact that blockade of E synthesis *in vivo* in the female rat affects the ability of luteal (current study) but not that of follicular granulosa cells [11] to produce progesterone on incubation with LH *in vitro*.

It is not clear if estrogen also regulates luteal function of the rabbit by a mechanism similar to the one described above. It is, however, known that blockade of E synthesis in the rabbit follicle, achieved by FSH antiserum administration, also results in inhibition in luteal P_4 production [33]. Block of E action during the luteal phase of the cycling monkey brought about with tamoxifen, a potent antiestrogen [34] or an E_2 antiserum [35] or inhibition of luteal E synthesis *in vivo* or *in vitro* by using the aromatase inhibitor [30] (CGS 16949A) does not result in suppression of P_4 secretion indicating that E perhaps has no role in regulating luteal function of the primate.

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