Progesterone Production *In Vitro* by Mouse Luteal Cells: Response to Follicle-Stimulating Hormone, Luteinizing Hormone, and Prolactin (44095)

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Abstract. **The purpose of this study was to determine the effects of ovine follicle-stimulating hormone (FSH), luteinizing hormone (LH); prolactin, and recombinant FSH and a protein kinase C activator (phorbol 12-myristate 13-acetate [PMA]) on progesterone production by dispersed luteal cells (large + small) from Day 4 pregnant mice. Corpora lutea (CL) were collected on Day 4 of pregnancy (Day 1 = sperm positive smear), and dispersed luteal cells were isolated using collagenase. After overnight incubation, the luteal cells were incubated with or without FSH, LH, prolactin, or recombinant human FSH or PMA for 4 hr or an additional 24 hr at 37°C; media were collected and progesterone was determined by RIA. Ten nanograms and 100 ng of ovine FSH, LH and prolactin were all equally effective in stimulating progesterone synthesis in media recovered after 24 hr of incubation. Moreover, the combination of all three gonadotropins yielded maximum levels of progesterone indicating a luteotrophic complex *in vitro*, paralleling previous *in vivo* findings. Recombinant human FSH—devoid of LH contamination—at doses of 10 and 100 ng also significantly stimulated progesterone synthesis, which strongly suggests that FSH has luteotropic activity in the mouse, thus agreeing with our previous *in vitro* results with CL of the pregnant hamster and rat. One hundred nanomolar PMA by itself did not affect progesterone production but significantly decreased dibutyrl cAMP-, forskolin-, FSH-, and LH-induced progesterone production, suggesting that activation of protein kinase C may block the luteotropic effects of LH and FSH during murine pregnancy.**

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Concurrent administration of prolactin and follicle-stimulating hormone (FSH) to the hypophysectomized, pregnant mouse maintains functional corpora lutea (CL) and pregnancy; either hormone by itself is ineffective (1). Thus, prolactin and FSH apparently constitute the minimal luteotropic complex. Substituting 1–100 ng luteinizing hormone (LH) for FSH failed to maintain pregnancy (1). With the use of a similar model, the minimal luteotropic complex maintains pregnancy in the hypophysectomized hamster (2, 3) and rat (4).

The luteotropic roles of prolactin (5) and LH (6) in rodents have long been recognized, but there has been a reluctance to grant similar status to FSH. This is mainly because of the indirect, *in vivo* approach and the possibility that LH contamination may account for some of the luteotropic activity of FSH. These potential problems have been eliminated in recent *in vitro* studies utilizing dispersed luteal cells from the pregnant hamster (7, 8) and rat (9). Moreover, the availability of recombinant human FSH, devoid of LH activity, makes it feasible to eliminate LH contamination as a confounding factor.

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The mouse is used extensively in reproductive studies, and we were therefore interested in determining whether our *in vitro* experiments in the hamster and rat would apply to this species. Because it is unusual for one laboratory to work with all three species using the same protocols and radioimmunoassay, we have included some of our previous results for comparative evaluation with luteal function in the mouse.

Protein kinase C (PKC) is an important second messenger protein for $PGF_{2\alpha}$ (10) and its effects on luteal cells have been investigated in hamster (7), rat (9), sheep (10), cow (11), and pig (12). However, there is little information about the effect of PKC on mouse luteal cells. This experiment, therefore, used luteal cells from pregnant mice to study the effects of PKC on steroidogenesis.

Materials and Methods

Cell Isolation. Adult CD-1 female mice (Charles River Laboratories, Raleigh, NC) were mated with fertile males of the same strain. Mice were checked for the presence of vaginal plugs (Day 1 of pregnancy). Mice on Day 4 of pregnancy were sacrificed between 0830 and 1000 hr, and ovaries were removed and immediately placed in ice-cold Hanks' balanced salt solution without Ca^{++} and Mg^{++} (Sigma Chemical Co., St. Louis, MO). Intact CL were dissected from the ovaries and placed in the enzyme solution consisting of 2.4 U/ml dispase (Grade II, Ca^{++} and Mg^{++}-free; Boehringer Mannheim Biochemicals, Indianapolis, IN), 0.1 mg deoxyribonuclease Type I and 50 IU collagenase (Type IV, Sigma). For each experiment, CL were pooled from four or five pregnant mice. Dissociation was carried out with sequential incubations (4 × 30 min) at 37°C in a shaking water bath. The CL were mechanically and gently agitated several times during each incubation. Following each 30-min period, the enzyme solution was decanted. Aprotinin (1 μg/ml, protease inhibitor; Sigma) was added to the enzyme solution containing luteal cells, which was then centrifuged (100g) for 10 min. The supernatant was discarded following centrifugation and fresh incubation medium was added to the luteal cells (1:1 McCoys [Life Technologies Inc., Grand Island, NY] and Ham's Nutrient Mixture F-12 [JRH Biosciences, Lenexa, KS] with 1% ITS [a combination of insulin, transferrin, selenium, and BSA; Becton Dickinson Labware, Bedford, MA], 100 IU penicillin/ml, and 100 mg streptomycin/ml). The washing procedure was repeated three times until the final incubation medium, containing cells, looked clear.

Immediately after enzymatic dissociation, large luteal cells (>20 μm) and small luteal cells (<20 μm) were counted with a hemocytometer. Approximately 2000 cells (large and small) were counted in duplicate for each of three separate experiments. The mean percentage of each cell type was then determined (Table I).

Table I. Percentage of Large (>20 μm) and Small (<20 μm) Cells on Day 4 of Pregnancy in Mouse, Hamster, and Rat

		%	
Species	Number of animals	Large (>20 μm)	Small (<20 μm)
Mouse	39	16.1 ± 0.55[a]	83.9 ± 0.92[b]
Rat[c]	38	15.2 ± 0.92[a]	84.6 ± 0.91[b]
Hamster[d]	42	4.0 ± 0.30[b]	96.0 ± 0.29[a]

[a,b]Percentages without a common letter are significantly different ($P < 0.05$).
[c]From Yuan and Greenwald (9).
[d]From Yuan and Greenwald (7).

Following isolation, cell numbers in the incubation medium were adjusted to 100,000 luteal cells (large + small cells) per ml and plated in 24-well plates (Costar, Cambridge, MA) at 1.0 ml/well with 10% bovine calf serum. The plates were placed overnight (16–20 hr) in an incubator with 95% air/5% CO_2, 100% humidity at 37°C. After overnight incubation, the media in the plates were discarded and 1 ml of fresh incubation media containing 5% Nutridoma NS (consisting of low density lipoprotein, transferrin, BSA, vitamins, amino acids, and insulin; Boehringer Mannheim Biochemicals, Indianapolis, IN) plus ovine 10 ng FSH, LH, and PRL, or a combination of these hormones, or Gonal-F recombinant human FSH (Ares-Serono's rhFSH; Ares Advanced Technology Inc, Randolph, MT) was added. The ovine hormones were provided by the National Hormone and Pituitary Program (Rockville, MD): FSH (S19), LH (S25), PRL (S19). The cells were incubated for 4 hr, the media decanted, and then incubated with added fresh hormones for an additional 24 hr. The dose response of ovine FSH, LH, and PRL (oFSH, oLH, and oPRL) at 0, 1, 10, and 100 ng on progesterone synthesis was tested first, and 10 ng of each hormone was selected as the definitive dose.

The hormone we were most interested in evaluating as a luteotropin was FSH. Ovine FSH-19 is an extremely potent preparation with 94× the biological potency of FSH-S1 while still possessing some LH activity (0.025 × LH-S1). For this reason, beyond Experiment 1, we decided not to test the effects of 100 ng of each hormone—alone or combined—but instead we relied on the recombinant human FSH—devoid of LH activity—for the definitive experiment.

To determine the effects of PKC on progesterone production, luteal cells were treated with 10 n*M* phorbol 12-myristate 13-acetate (PMA) with or without 10 μ*M* dibutyrl cAMP (dbcAMP), 1 m*M* forskolin (all from Sigma), FSH, or LH for 4 hr. The doses of dbcAMP and forskolin were based on our previous experiments

with hamster (7, 8) and rat (9). The collected media were stored at −20°C. Progesterone was determined by radioimmunoassay (RIA). For each hormone or combination tested, duplicate samples of luteal cells were run in each incubation; there were three replicates for each treatment.

Following enzymatic isolation, luteal cell viability was checked using 0.4% trypan blue exclusion; viability was between 83%–87%. When the cells were removed from the culture plates after overnight incubation, cell viability was checked again and was 76%–79% and 72%–75% at 4 and 24 hr of incubation, respectively.

Progesterone Determination and Statistical Analysis. The concentration of progesterone in the incubation medium was determined by RIA, without extraction, using progesterone antiserum developed in rabbits (13). Labeled [1,2,6,7-^{3}H] progesterone was purchased from Dupont (Boston, MA). The mean sensitivity of the assay at 95% binding was 5 pg/tube. Progesterone concentration was expressed as ng/100,000 cells.

The data were analyzed by a randomized complete block design. The analysis of variance (ANOVA) analysis was carried out with the SuperANOVA program of Abacus Concepts, Inc. (Berkeley, CA) using a Macintosh Classic II computer followed by Duncan's New Multiple Range test. Treatment effects were evaluated at each incubation time of each day. Differences at a probability of $P < 0.05$ were considered statistically significant.

Results

On Day 4 of pregnancy, mouse CL contained about four times as many large cells as the hamster but percentage of large cells similar to that in the rat (Table I).

Ovine FSH, LH, and PRL significantly increased progesterone synthesis by mouse luteal cells on Day 4 of pregnancy, in a dose-dependent manner, at a dose of 10 ng or 100 ng of each hormone (Fig. 1). It might be argued that the dosage intervals in Figures 1 and 2 are too large to generate accurate dose-response curves for the different hormones since biphasic effects of gonadotropins have been reported with high doses (100 ng/ml) being inhibitory. It is therefore possible that the optimal effective response occurred somewhere between 10 and 100 ng/ml. However, it is quite common to use ten-fold differences in hormone doses in establishing dose-response relationships. Moreover, on Day 4 of murine pregnancy, peripheral plasma levels of progesterone are approximately 25 ng/ml (14). We therefore felt justified in utilizing 10 ng/ml of the hormones in the definitive experiment in Figure 3. Recombinant human FSH—without LH activity—also stimulated progesterone production at 10 or 100 ng (Fig 2) but the 100-ng dose was not as effective as 100 ng of ovine FSH (Fig. 1). Based on the results in Figure 1, 10 ng of each ovine

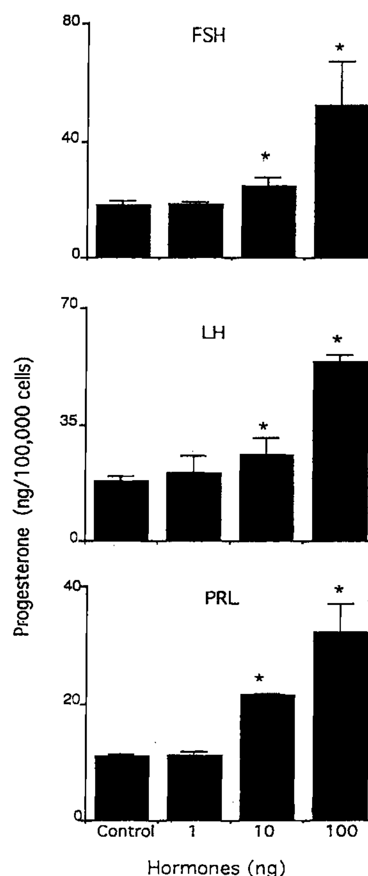

Figure 1. Effects of 1, 10, and 100 ng ovine FSH (upper), LH (middle), and prolactin (lower) on progesterone synthesis by mouse luteal cells on Day 4 of pregnancy after 24 hr of incubation. Note the differences in the *y* axis for each hormone. In all experiments, there was 1 ml of medium/well. *Significantly different from control ($P < 0.05$). *Note.* In Figures 1 through 4, the values represent the mean ± SEM of duplicate determination from three separate experiments.

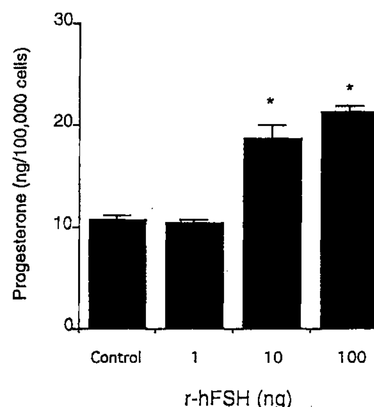

Figure 2. The dose-response effects of human recombinant FSH (r-hFSH) on progesterone synthesis by mouse luteal cells on Day 4 of pregnancy following 24 hr of incubation. *Significantly different from control ($P < 0.05$).

hormone was selected to test possible interactions on progesterone synthesis. The three hormones alone or any possible combination of two hormones significantly

increased progesterone at 4 or 24 hr of incubation (Fig. 3), but the latter regimens were no more effective than the single gonadotropins. However, combined exposure to FSH + LH + PRL significantly enhanced progesterone synthesis at both 4 and 24 hr of incubation (Fig. 3). Thus, additive effects were elicited when all three hormones were combined *in vitro*.

One hundred nanomolar PMA by itself did not affect progesterone secretion (Fig. 4) but significantly decreased dbcAMP-, forskolin-, FSH-, and LH-induced progesterone production.

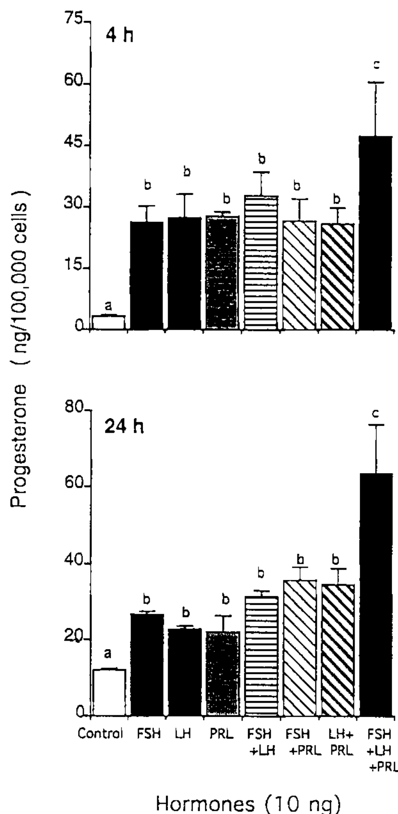

Figure 3. Effects of 10 ng ovine FSH, LH, and prolactin, or various combinations of the hormones, on progesterone synthesis by mouse luteal cells on Day 4 of pregnancy at (a) 4 hr of incubation, (b) 24 hr of incubation. The values without a common letter are significantly different ($P < 0.05$). *Note.* oFSH + LH + PRL stimulated progesterone more than any other treatment ($P < 0.05$).

Discussion

On Day 4 of pregnancy, the corpora lutea of the mouse and rat contain about four times as many large luteal cells as the hamster (Table I), which correlates with the amount of progesterone secreted *in vitro* in control incubations (Table II). This suggests that the bulk of progesterone in dispersed luteal cells from the mouse and rat comes from large luteal cells, consistent with the findings in domestic species (10, 5).

It might be argued that on Day 4 of pregnancy mouse luteal cells are still undifferentiated granulosa cells and therefore still retain persistent FSH receptors. Several lines of evidence negate this possibility. By Day 4 of pregnancy, the murine CL has increased in diameter to 684 μm, compared with 384 μm on Day 1; this correlates histologically with the presence of luteal cells with a further increase in the cytoplasmic-nuclear ratio compared with Day 3 (16). In agreement with these findings, plasma progesterone is low on Days 1 and 2 (1–2 ng/ml), whereas peripheral levels are approximately 25 ng/

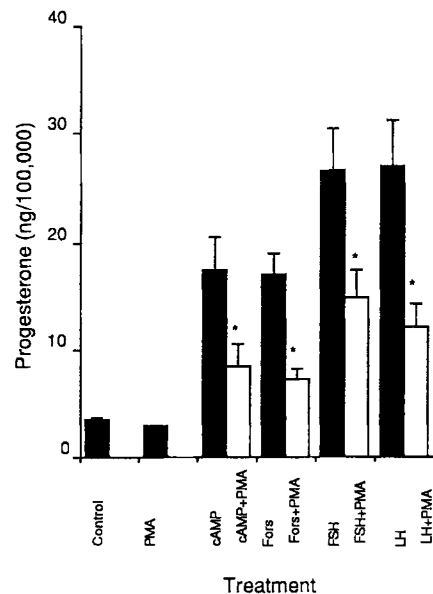

Figure 4. Effects of 100 nM PMA on 10 μm cAMP-, 1 m*M* forskolin-, 10 ng FSH-, and 10 ng LH-induced progesterone production by mouse luteal cells on Day 4 of pregnancy at 4 hr of incubation. *Significantly different from controls ($P < 0.05$).

Table II. Effects of 10 ng Ovine FSH, LH, or Prolactin in 1 ml of Medium on Progesterone Production (ng/100,000 cells; mean ± SEM) by Mouse, Rat, and Hamster Luteal Cells on Day 4 of Pregnancy after 24-hr Incubation

Species	Control	FSH	LH	PRL	FSH + LH + PRL
Mouse	11.9 ± 0.5	26.7 ± 0.5	22.6 ± 0.8	21.6 ± 4.2	63.6 ± 12.9
Rat[a]	37.4 ± 6.2	66.6 ± 6.0	56.9 ± 3.2	59.9 ± 6.1	155.4 ± 2.8
Hamster[a]	2.8 ± 0.6	6.6 ± 2.2	9.1 ± 1.1	2.8 ± 1.0	14.4 ± 3.0

[a]From Yuan and Greenwald (9).
[b]From Yuan and Greenwald (7).

Table III. Effects of Recombinant Human FSH in 1 ml of Medium on Progesterone Production (ng/100,000 cells, mean ± SEM) by Mouse, Rat, and Hamster Luteal Cells during 24-hr Incubation on Day 4 of Pregnancy

Species	0 ng	1 ng	10 ng	100 ng
Mouse	10.7 ± 0.5	10.4 ± 0.3	18.7 ± 1.4[a]	21.4 ± 5.4[a]
Rat[b]	33.5 ± 4.9	32.9 ± 6.4	84.7 ± 21.4[a]	96.8 ± 23.1[a]
Hamster[c]	4.6 ± 1.1	5.1 ± 1.3	6.8 ± 1.1	11.3 ± 2.0[a]

[a]Significantly different from Control ($P < 0.05$).
[b]From Yuan and Greenwald (9).
[b]From Yuan and Greenwald (7).

ml on Day 4 (14). Hence, by structural and functional criteria these are *true* luteal cells. Furthermore, CL of cyclic mice show, by autoradiography, binding sites for hCG but not FSH (17), whereas FSH receptors are present in CL of hypophysectomized gonadotropin-treated mice on Day 2 but not on Day 1 of pregnancy (18). Although we did not test the *in vitro* responsiveness of luteal cells to FSH in the mouse and rat beyond Day 4 (9), the luteotropic activity of FSH is demonstrable by dissociated hamster luteal cells on Day 12 (7).

In all three rodent species, ovine FSH significantly increased progesterone to the same extent as LH and PRL, except for the hamster, where PRL was not effective on Day 4 (Table II). On Day 12, the percentage of large luteal cells in the hamster increase to 10.7%, which correlates with a now significant luteotropic response to prolactin (7). If the species are ranked according to *in vitro* production of progesterone in basal incubations or in the presence of any of the gonadotropins, the sequence is rat > mouse > hamster (Table II). We deliberately limited outselves to 10 ng in testing the *in vitro* luteotropic effects of the hormones to minimize the amount of LH bioactivity present in ovine FSH (250 pg of LH in 10 ng FSH), which is a rather inconsequential amount. It is noteworthy that recombinant human FSH without any LH contamination was able to increase progesterone production in all three species but was effective at a lower dose in the mouse and rat (Table III). This again points out that FSH possesses luteotropic activity in pregnant rodents.

Although FSH has rarely been considered to be a luteotrophic hormone, scattered references suggest that it may be of more universal significance. For example, FSH receptors are present in the hamster (19), cow (20), and human corpus luteum (21). *In vitro* luteotrophic action of FSH is demonstrated by porcine luteal cells during the early stages of the estrous cycle (22). Injecting heifers on Days 10–23 of the estrous cycle with oxytocin increases serum FSH but not LH, PRL, or estradiol; extends luteal life span; and prolongs progesterone synthesis (23).

In the present study, the highest level of progesterone synthesis occurred when combined FSH, LH, and PRL were added to the dispersed luteal cells, and this was also true for the rat and hamster (Table III). Correspondingly, FSH + LH + PRL is the best *in vivo* treatment to restore all luteal parameters and maintain pregnancy (3, 4, 24). Thus, a luteotropic complex—at least in the rodents—seems essential, *in vitro* and *in vivo*, to maintain optimal luteal function.

Many studies have demonstrated that PMA is an activator of PKC (10, 11). By itself, PMA did not affect progesterone secretion. This finding was different from that of other studies, in which PMA alone decreased progesterone production (10). It is not clear why PMA can directly inhibit progesterone secretion by ovine luteal cells from cyclic animals but not by luteal cells from pregnant animals. Interestingly, PMA decreased dbcAMP-, forskolin-, FSH-, and LH-induced progesterone production by mouse luteal cells from pregnant animals. The inhibitory effects of PMA on progesterone in the mouse were similar to the effects of PMA in the hamster and rat during pregnancy (7, 9). This suggests that PKC can block progesterone production by LH or FSH during pregnancy. Activation of protein kinase C by PMA inhibits P_{450} cytochrome side chain cleavage in ovine large luteal cells (10), as measured by metabolism of 25-hydroxycholesterol (25). In the present study, therefore, the decrease in progesterone synthesis by PMA plus LH or FSH is likely due to inhibition of steroidogenic enzymes.

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