PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 170, 225–230 (1982)

Is the First or Second Periovulatory Surge of FSH Responsible for Follicular Recruitment in the Hamster? (41423)

GILBERT S. GREENWALD AND HAROLD I. SIEGEL

Department of Physiology, Ralph L. Smith Research Center, University of Kansas Medical Center, Kansas City, Kansas 66103

Abstract. Proestrous hamsters were injected at 1300 hr with phenobarbital to block ovulation. The animals were bled via cardiac puncture every 6 hr beginning at 1600 hr of proestrus until 1000 hr of estrus. The ovaries were then removed and a quantitative evaluation of follicular development carried out. In saline-injected proestrous hamsters, there was a peak of serum FSH and LH at 1600 hr followed by a second prolonged peak of FSH at 0400 and 1000 hr of estrus accompanied by low tonic levels of LH. This hormone profile was associated at 1000 hr of estrus with an average of 9.3 large preantral follicles (stage IV follicles) per ovary. In contrast, the phenobarbital-treated hamsters showed no increases in either gonadotropin at any time and the ovaries contained large antral follicles and an average of only 1.7 stage IV follicles per ovary (three of four animals). Injection of 1 mg progesterone concurrent with phenobarbital restored the first surge of LH but not FSH and partially restored the second peak of serum FSH; the ovaries contained newly formed corpora lutea and 10.5 stage IV follicles per ovary. Injection of 1 μg estradiol along with phenobarbital did not restore the first peak of LH or FSH and partially restored the second FSH surge; the ovaries contained large Graafian follicles but also an average of 10.8 stage IV follicles. Concurrent injection of progesterone and estradiol at the same time as phenobarbital restored the second FSH peak to the magnitude of control values and again the ovaries contained 10.8 stage IV follicles. By using the same animals for periovulatory measurements of gonadotropins and for ovarian histology, these experiments clearly demonstrate that the second prolonged release of FSH is responsible for the recruitment at estrus of the large preantral (stage IV) follicles which represent the pool from which the follicles ovulating 4 days later are selected.

The hamster shows a very distinct biphasic pattern of serum FSH during the periovulatory period with the first surge associated with the preovulatory increase in LH whereas the second FSH peak extends through most of estrus and is accompanied by low, tonic levels of LH (1). In a subsequent study (2) we delineated the exact times when these events occurred, with the first FSH and LH surges lasting from 1400 to 1800 hr on proestrus (Day 4 of the cycle) followed by serum FSH and LH returning to basal levels by 2200 hr and a second more sustained surge of FSH between 2400 and 0800 hr of estrus (Day 1 of the cycle). This discrete pattern of two FSH peaks in the hamster differs from the periovulatory profile in the rat in which once FSH levels rise at proestrus, they are maintained without a drop throughout the morning of estrus (3, 4).

In the hamster, the follicles destined to ovulate at the next cycle can first be distinguished on the morning of estrus (Day 1 of the cycle) as large preantral follicles with 8–12 layers of granulosa cells (5, 6). Moreover, in long-term hypophysectomized hamsters the largest preantral follicles present have no more than 5 layers of granulosa cells and an initial large sc dose of FSH (200 μg) is required followed by 50 μg FSH a day for 3 days and then 10 μg of LH (ip) to induce ovulation of nine eggs—comparable to the ovulation rate of intact hamsters (7). Thus, either a normal or artificial surge of FSH is necessary for the development of large preantral follicles in the hamster ovary.

We have previously reported that the injection of phenobarbital at 1300 hr on Day 4 of the hamster cycle blocks not only ovulation by suppressing LH release but also the two FSH peaks (2). However, injection of progesterone or estrogen concurrent

0037-9727/82/060225-06$01.00/0

with phenobarbital restores the second FSH peak. This ability to manipulate whether there are one or two FSH peaks combined with quantitative evaluation of follicular development (6) on the morning of estrus enables us to resolve whether the first and/or second peak of FSH is essential for recruitment of the follicles at estrus which will ovulate 4 days later.

Materials and Methods. Golden hamsters (80–120 g) were maintained on a 14-hr light:10-hr dark regimen (lights on:0500 hr). Before being used in the experiment, all animals had at least three consecutive 4-day cycles which were determined by monitoring the postovulatory vaginal discharge present on the morning of estrus (Day 1); proestrus corresponds to Day 4. Hamsters (four in each group) were injected sc at 1300 hr of Day 4 with phenobarbital (Phen) (6.5 mg/100 g body weight) and 0.1 ml (ip) of progesterone and/or estradiol at the dosages listed in Table I. As a control, four hamsters were injected sc at 1300 hr Day 4 with 0.1 ml saline and 0.1 ml sesame oil (ip).

Beginning at 1600 hr and at 6-hr intervals thereafter each unanesthetized animal was bled by cardiac puncture (using 27-gauge needles) and the serum saved for RIA of FSH and LH. After the last bleeding on Day 1 at 1000 hr, the animals were killed with an overdose of ether, the oviducts flushed to recover newly ovulated ova, and one ovary from each animal preserved in Bouin's solution for histology (see below). Fifty or 100 microliters of serum were diluted to a constant volume of 250 μl with 1% (w/v) egg white in 0.01 *M* phosphate-buffered saline and stored at −12° until assayed for LH and FSH.

The general methodology for the RIAs has been previously described (1). The ovine:ovine LH RIA system (8) and the rat:rat FSH RIA system were used. The final incubation volume in an assay tube was 0.5 ml instead of 1.0 ml and was obtained by reducing the volume of each component in the final incubation solution by 50%. For FSH, NIAMDD anti-rat FSH-6 was used and serum hormone levels were expressed in terms of NIAMDD rat FSH RP-1 (2.1 × NIH-FSH-S1). For LH, anti-ovine LH serum (GDN-15) and an ovine LH for iodination (LER-1056-C2) were provided by Drs. Gordon D. Niswender and Leo E. Reichert, respectively. Serum hormone levels of LH were expressed in terms of NIAMDD rat LH-RP-1 (0.03 × NIH-LH-S1).

To quantify follicular development the ovaries removed at 1000 hr on Day 1 were prepared for light microscopy by standard procedures. The ovaries were sectioned serially at 10 μm and stained with hematoxylin and eosin. Follicles were counted in every fourth section at a magnification of 100× (6). Only healthy nonatretic follicles in which the nucleolus of the oocyte was present were counted with the exception that all antral follicles were counted whether the nucleolus was present or absent in the sections that were scanned. Follicular development was divided into six stages:

- Stage I preantral follicles consisting of 2–3 layers of granulosa cells;
- Stage II preantral follicles with 4–5 layers of granulosa cells;
- Stage III preantral follicles with 6–7 layers of granulosa cells;
- Stage IV preantral follicles with 8–12 layers of granulosa cells;
- Stage V follicles with about 12 layers of granulosa cells and with incipient antral formation as evidenced by a series of isolated lacunae in the granulosa cells;
- Stage VI antral follicles with a single coalesced cavity.

Contrary to the previous study (6), stage I follicles were not counted to reduce the tedious task of counting small preantral follicles which were not germane to the purpose of the investigation. To avoid introducing bias all ovaries were coded and read as unknowns and statistical analysis was deferred until all ovaries had been read. Statistical significance was established by the Student's *t* test.

Results. In confirmation of our previous results (2) the injection of phenobarbital at 1300 hr of Day 4 (proestrus) blocked ovula-

tion in all treated animals and ovulation was restored by progesterone but not estrogen as attested by the presence or absence of large antral follicles (stage VI) on Day 1 of the next cycle (Table I).

The control group (group 1) showed the biphasic FSH peaks at 1600 hr of Day 4 and 0400 hr of Day 1 and this was associated with the presence of large preantral follicles (stage IV) on the morning of Day 1. In contrast, a single injection of Phen blocked ovulation, the FSH and LH peaks and the ovaries contained only a few stage IV follicles (group 2), stage IV follicles were present in the ovaries of only three of the four animals in the group. It is noteworthy that in group 2, there were also reduced numbers of follicles in stages II and III. Concurrent injection of progesterone along with Phen restored the LH peak but not the first FSH peak at the times studied; however, an increase in serum FSH was found on Day 1 at 0400 and 1000 hr albeit at lower levels than in the control group (cf. groups 1 and 3). It is noteworthy that the restored second FSH peak led to the appearance on the next morning of stage IV follicles (group 3).

The phenobarbital block of ovulation was not reversed by 1 μg estradiol (or 10 μg estradiol; data not shown in Table I) but the restoration of the second FSH peak again led to the normal number of stage IV follicles on Day 1 (group 4). The combination of progesterone and estradiol to the Phen-treated hamsters (group 5) restored the second serum FSH peak to the same values as the controls and also recruited stages II–IV follicles to the same range in numbers as in the saline-injected controls (group 1).

For all of the groups which ultimately ovulated (groups 1, 3, 5), the serum LH levels at 1600 hr Day 4 were too high to be quantitated with 50 μl of serum. From Table I it is also evident that the second peak of FSH was never accompanied by similar dramatic increases in serum LH.

Discussion. These experiments demonstrate that by suitable manipulations, it is possible to block the normal serum profiles of serum FSH and LH in the periovulatory period (Table I, group 1). Thus, it is feasible to block all of the normal increases in serum gonadotropins by phenobarbital (group 2) and to selectively restore the second FSH surge by either progesterone or estradiol (groups 3–5) with or without the first LH surge (groups 3, 5 versus group 4). The unique feature of these experiments is that it is the only study which combines gonadotropin determinations in the periovulatory period with the same animals used for detailed quantitative evaluation in the early postovulatory period.

From this standpoint, the results in Table I clearly demonstrate that only the second FSH peak is needed to recruit the large preantral follicles (stage IV) which will ovulate 4 days later. In the presence of only tonic levels of FSH-LH in the periovulatory period—which is induced by phenobarbital—there are significant differences on Day 1 in the numbers of stage II and IV follicles although in three of four animals there are still an average of 1.7 stage IV follicles per ovary (group 2). However, all other steroid treatments superimposed on phenobarbital recruited stage IV follicles to the same extent as in the control group (group 1). Groups 3 and 4 demonstrate that on Day 1 at 0400 hr considerably less serum FSH (184–221 ng/ml) than normal is sufficient to mature large preantral follicles. It is noteworthy that neither the first LH or FSH peaks are needed to reverse the effects of phenobarbital on follicular development (group 4).

Treatment with phenobarbital alone also reduced the number of stage II and III follicles in addition to affecting the stage 4 group (Table I, group 2). In hamsters hypophysectomized for 5 days, the ovaries contain significantly fewer stage I–III follicles (7, 9) than the ovaries of intact cyclic hamsters (5) and in the former group, there are no follicles in stages IV–VI. When hypophysectomized hamsters were given a single sc injection of 100 μg FSH and killed 11 hr later, the ovaries contained significantly more stage I–IV follicles than controls (9). Thus, in the present study, the absence of the second FSH surge in the phenobarbital-blocked animals was reflected in a paucity of follicles in all of the

TABLE I. CORRELATION OF PERIOVULATORY SERUM LEVELS OF FSH AND LH AND FOLLICULAR DEVELOPMENT AT ESTRUS (DAY 1)[a]

Treatment at 1300 hr Day 4	Hormone	Serum level of FSH or LH (ng/ml ± SEM) at: Day 4 1600 hr	Day 4 2200 hr	Day 1 0400 hr	Day 1 1000 hr	Follicular development per ovary on Day 1: Stage[b]	No. of follicles per stage ± SEM
Controls	FSH	701 ± 27	240 ± 16	619 ± 27	570 ± 41	II	35 ± 4.4
						III	12.8 ± 3.1
(Group 1)	LH	>128	20 ± 2	14 ± 2	19 ± 3	IV	9.3 ± 2.3
						V	3.5 (2)
						VI	—
Phen	FSH	165 ± 17*	262 ± 39	272 ± 42*	236 ± 5*	II	15.5 ± 3.2*
						III	6.5 ± 1.2
(Group 2)	LH	32 ± 6*	31 ± 10	29 ± 3*	24 ± 6	IV	1.7 ± 0.6 (3)**
						V	2.3 ± 0.7
						VI	5.8 ± 0.9
Phen + 1 mg P_4	FSH	165 ± 41*	158 ± 24	435 ± 29*	415 ± 14*	II	36 ± 3.8
						III	10 ± 2.1
						IV	10.5 ± 2.8
(Group 3)	LH	>128	20 ± 3	24 ± 11	24 ± 5		
Phen + 1 μg E_2	FSH	179 ± 30*	249 ± 34	398 ± 38*	364 ± 102	II	30.3 ± 2.6
						III	12.8 ± 1.6
						IV	10.8 ± 1.5
(Group 4)	LH	24 ± 8*	27 ± 15	10 ± 1	13 ± 5	V	-0-
						VI	5.3 ± 0.8
Phen + P_4 + 1 μg E_2	FSH	390 ± 49*	231 ± 56	519 ± 35	502 ± 57	II	34.3 ± 2.9
						III	12.3 ± 1.8
						IV	10.8 ± 2.4
(Group 5)	LH	>163	23 ± 8	24 ± 9	15 ± 2		

[a] There are four animals per group.

[b] Stage II = preantral follicles (PAF) with 4–5 layers of granulosa cells (GC); III = PAF with 6–7 layers GC; IV = PAF with 8–12 layers GC; V = incipient antral; VI = large antral follicles.

* $P < 0.01$ compared to corresponding control value (Group 1).

** $P < 0.02$, by Student's t test.

smaller stages of development which was reversed whenever the second FSH was restored.

In the interim since the present work was completed (1974) several papers have been published on the same theme but with different experimental designs. When proestrous rats ($n = 4$) were injected with phenobarbital at 1200 and 1500 hr and killed at estrus at 1200 hr, the ovaries contained only two "pre-Graafian follicles" which were between 390 and 500 μm in diameter compared to an average of 9.7 follicles per ovary in control animals (10). Injection of 500–600 μg of rat FSH at the time of the second injection of phenobarbital at proestrus resulted the next day in the ovaries containing 4.5 large preantral follicles per ovary (10). Similarly, in the hamster injection of phenobarbital at 1300 hr of proestrus resulted the next morning in the presence of only 2.5 stage IV follicles per ovary (11).

Another approach which has been employed to correlate periovulatory gonadotropin levels with follicular selection is the use of inhibin to preferentially alter serum FSH levels without affecting serum LH. For example, injecting hamsters every 3 hr with bovine follicular fluid (500 μl) beginning at 1800 hr proestrus until 0900 estrus results at the *next* cycle in the ovulation of only 5 ova instead of the normal number of 12.6 and starting the injections at 0900 hr of proestrus results in no ovulations at the next periovulatory period (12). This is associated with complete suppression of the second serum FSH peak. Similarly, in the rat after single or multiple injections (beginning at 1100 hr proestrus) of porcine follicular fluid the next morning the ovaries lack follicles greater than 400 μm in diameter (13). A previous study from this group showed that injections of porcine follicular fluid resulted in suppression of plasma FSH for 14–18 hr but by 0800 hr of estrus plasma FSH values were greater than 150 ng/ml in animals given a single injection at 1100 hr of proestrus (14).

Still another way of demonstrating the importance of the second FSH surge for follicular recruitment is by the injection of a long-acting esterified estrogen on Day 1 of the hamster cycle (15). Under these circumstances, at the subsequent proestrus and estrus the first LH-FSH surge takes place but the second FSH peak is blocked. The net result is that antral follicles do not reappear until 14 days after the estrogen treatment and ovulation does not recur until 18 to 22 days post-treatment (15).

Collectively, all of these studies demonstrate the importance of the FSH surge to launch the next set of follicles on the path to ovulation and in particular, the results summarized in Table I of this paper show the significance of the second FSH surge as the sine qua non for follicular recruitment. How then does FSH exert its effects? Presumably by affecting folliculogenesis at multiple levels including:

(i) Increased steroidogenic capacity of the stage IV hamster follicle; e.g., stage IV follicles on Day 1 of the cycle incubated for 1 hr produce progesterone and 17-hydroxyprogesterone as the major steroids compared to negligible amounts of testosterone and estradiol (16) and the steroids in turn may influence subsequent development of the follicles.

(ii) Increase in the number of LH receptors in granulosa cells (17) although at 0900 hr of Day 1 of the hamster cycle the stage IV follicles, by topical autoradiography, contain only FSH receptors and no LH binding sites (Oxberry and Greenwald, unpublished).

(iii) Granulosa cells from small preantral follicles from hypophysectomized rats treated with estradiol increase their production of cyclic AMP in response to FSH—not hCG (18). However, hamster stage IV follicles on Day 1 of the cycle produce *in vitro* the lowest level of cAMP of any time during the 4-day cycle (16). It is possible that factors i–iii may not be manifested until later stages of folliculogenesis.

(iv) Possibly all of the aforementioned actions of FSH culminate in the increased rate of ovarian thymidine incorporation on Day 1 of the cycle in the hamster ovary which largely reflects the percentage of labeled stage IV follicles as evidenced by autoradiography (19). Although emphasis in

this paper has been on the critical role of the second FSH surge in follicular selection, the low tonic levels of serum LH present throughout estrus may also be required for synergistic interactions with FSH. In the hamster administration of a potent equine anti-bovine LH serum at 0900 hr of Day 1 results the next morning in atresia of all stage III and IV follicles (20) which is correlated with normal serum levels of FSH but with serum LH below the detectable limits of the radioimmunoassay (21). Thus, the larger preantral follicles are already LH dependent.

This research was supported by a grant from NIHCHD-00590-20. We thank the NIAMMD program for providing the RIA kits.

1. Bast JD, Greenwald GS. Serum profiles of follicle-stimulating hormone, luteinizing hormone and prolactin during the estrous cycle of the hamster. Endocrinology **94**:1295–1299, 1974.
2. Siegel HI, Bast JD, Greenwald GS. The effects of phenobarbital and gonadal steroids on periovulatory serum levels of luteinizing hormone and follicle-stimulating hormone in the hamster. Endocrinology **98**:48–55, 1976.
3. Gay VL, Midgley AR Jr, Niswender GD. Patterns of gonadotropin secretion associated with ovulation. Fed Proc **29**:1880–1887, 1970.
4. Duane TA, Parlow AF. Periovulatory patterns of rat serum follicle stimulating hormone and luteinizing hormone during the normal estrous cycle: Effects of pentobarbital. Endocrinology **88**:653–663, 1971.
5. Greenwald GS. Quantitative study of follicular development in the ovary of the intact or unilaterally ovariectomized hamster. J Reprod Fertil **2**:351–361, 1961.
6. Greenwald GS. Quantitative aspects of follicular development in the untreated and PMS-treated hamster. Anat Rec **178**:139–143, 1974.
7. Moore PJ, Greenwald GS. Effect of hypophysectomy and gonadotropin treatment of follicular development and ovulation in the hamster. Amer J Anat **139**:37–48, 1974.
8. Niswender GR, Reichert LE Jr, Midgley AR Jr, Nalbandov AV. Radioimmunoassay for bovine and ovine luteinizing hormone. Endocrinology **84**:1166–1173, 1971.
9. Chiras DD, Greenwald GS. Effects of steroids and gonadotropins on follicular development in the hypophysectomized hamster. Anat Rec **152**: 307–319, 1978.
10. Hirshfield AN, Midgley AR Jr. The role of FSH in the selection of large ovarian follicles in the rat. Biol Reprod **19**:606–611, 1978.
11. Terranova PF. Effects of phenobarbital-induced ovulatory delay on the follicular population and serum levels of steroids and gonadotropins in the hamster: A model for atresia. Biol Reprod **23**:92–99, 1980.
12. Chappel SC, Selker F. Relation between the secretion of FSH during the periovulatory period and ovulation during next cycle. Biol Reprod **21**:347–352, 1979.
13. Hirshfield AN, DePaolo LV. Effect of suppression of the surge of follicle-stimulating hormone with porcine follicular fluid on follicular development in the rat. J Endocrinol **88**:67–71, 1981.
14. DePaolo LV, Hirshfeld AN, Anderson LD, Barraclough CA, Channing CP. Suppression of pituitary secretion of follicle-stimulating hormone by porcine follicular fluid during proestrus and oestrus in the rat: effects on gonadotrophin and steroid secretion, follicular development and ovulation during the following cycle. J Endocrinol **83**:355–368, 1979.
15. Greenwald GS. Proestrous hormone surges dissociated from ovulation in the estrogen treated hamster. Endocrinology **97**:878–884, 1975.
16. Hubbard CJ, Greenwald GS. Cyclic nucleotides, DNA, and steroid levels in ovarian follicles and corpora lutea of the cyclic hamster. Biol Reprod **26**:230–240, 1982.
17. Richards JS, Midgley AR Jr. Protein hormone action: A key to understanding ovarian follicular and luteal development. Biol Reprod **14**:82–94, 1976.
18. Richards JS, Jonassen JA, Rolfes AI, Kinsey K, Reichert LE Jr. Adenosine 3′5′ monophosphate, luteinizing hormone receptor, and progesterone during granulosa cell differentiation: Effects of estradiol and follicle stimulating hormone. Endocrinology **104**:765–773, 1979.
19. Chiras DD, Greenwald GS. Analysis of ovarian follicular development and thymidine incorporation in the cyclic golden hamster. Amer J Anat **157**:309–317, 1980.
20. Greenwald GS, Terranova PF. Induction of superovulation in the cyclic hamster by a single injection of antiluteinizing hormone serum. Endocrinology **108**:1903–1908, 1981.
21. Terranova PF, Greenwald GS. Alteration of the serum follicle-stimulating hormone to luteinizing hormone ratio in the cyclic hamster treated with anti-luteinizing hormone: Relationship to serum estradiol, free anti-luteinizing hormone, and superovulation. Endocrinology **108**:1909–1914, 1981.

Received November 10, 1981. P.S.E.B.M. 1982, Vol. 170.