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Alterations in Follicular Fluid Steroids and Follicular hCG and FSH Binding during Atresia in Hamster (42074)

JOONG YOL NA, FRANK GARZA,[1] AND PAUL F. TERRANOVA

Department of Physiology, Ralph L. Smith Research Center, University of Kansas College of Health Sciences and Hospital, Kansas City, Kansas 66103

Abstract. Preovulatory follicles from hamsters treated on proestrus for 1–3 days with phenobarbital sodium exhibited early signs of atresia after 2–3 days of ovulatory delay. A significant increase in follicular fluid progesterone was evident by Day 1 of delay. Concentrations of androstenedione in follicular fluid were unaffected by ovulatory delay. Follicular fluid levels of estradiol in delayed follicles were either higher than proestrous values after 1 day of delay or lower after 2 and 3 days of delay. hCG binding was slightly higher than proestrous controls after ovulatory delay whereas FSH binding was significantly lower than controls after 2 and 3 days of ovulatory delay. These results indicate that in the barbiturate-treated hamster the elevated follicular fluid levels of progesterone precede by 1–2 days the previously reported increase in steroidogenic capability of delayed follicles to produce progesterone *in vitro;* this correlated with an increase in the ratio of hCG:FSH binding and this was mostly due to a decrease in FSH binding to whole follicles.

Previous studies have used barbiturate-delayed ovulation as a model for studying atresia of preovulatory follicles in rats and hamsters (1–4). As the follicles enter the atretic phase (Days 2–3 of delay), an increase in the *in vitro* capability to produce progesterone in response to LH was observed; in addition the delayed-atretic follicles exhibited a reduced capacity to produce androstenedione and estradiol *in vitro.* In the 5-day cyclic rat, hCG and FSH binding to granulosa cells of delayed follicles declined after 2 days of ovulatory delay, although an increase in only hCG binding was observed after 1 day of delay.

Follicular progesterone, androstenedione, testosterone, estrone, and estradiol levels have been determined in the 4-day cyclic rat after 1 and 2 days of ovulatory delay (5). Testosterone, androstenedione, and estradiol levels in follicles were ≃200–300 pg/follicle on the morning of proestrus in the 4-day cyclic rat; whereas 2 days of ovulatory delay increased the follicular estradiol level to ≃500 pg/follicle without alteration in the progesterone, androstenedione, and testosterone levels. The endogenous follicular steroid levels contrast somewhat with the previously described *in vitro* estradiol to progesterone shift in steroid production of delayed follicles. Thus, it appears that the *in vitro* steroidogenic capability of follicles did not reflect endogenous levels of follicular steroids; however, this must be interpreted with caution since the data have been collected from several studies using different strains of rats with either 4- or 5-day cycles. In order to ascertain whether the estradiol–progesterone shift observed *in vitro* could be also observed *in vivo,* the present investigation was conducted using the 4-day cyclic hamster. The present study was designed to examine the temporal relationships among follicular fluid steroid levels during barbiturate-induced ovulatory delay, relate this to hCG and FSH binding, and to the previously described *in vitro* estradiol to progesterone shift (4, 6).

Materials and Methods. Cyclic hamsters weighing 80–110 g were obtained from SASCO, Inc. (Omaha, Nebr.) and maintained in 22°C rooms with a 14 L:10 D daily lighting schedule (lights on at 0500 hr). Hamsters were used following three consecutive 4-day cycles. In order to delay ovulation 1 day, hamsters were injected sc at 1300 hr proestrus with 9.75 mg phenobarbital sodium/100 g body wt; hamsters injected the next day at 1200 hr with 13 mg phenobarbital/100 g

[1] Present address: Department of Animal Science, Louisiana State University, Baton Rouge, La. 70803.

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body wt exhibited a 2-day delay; an additional injection of 19.5 mg phenobarbital/100 g body wt at 1200 hr on the following day was required for 3 days of ovulatory delay. An earlier study established the injection schedule for blockade of ovulation (3). Hamsters blocked with phenobarbital on proestrus (Day 4) exhibited LH and FSH surges on Day 5 and ovulation on Day 6; a 2-day blockade postponed ovulation until Day 7; with 3 days of blockade, ovulation occurred on Day 8. Histologic examination of the ovary revealed normal preovulatory follicles after 0–2 days of delay. However, on Day 3 of delay the original set of preovulatory follicles exhibited early signs of atresia (3).

In order to study the effect of ovulatory delay on the steroid concentrations of the follicular fluids and on the FSH and LH receptors of the follicles, 2 groups of 32 hamsters each were used (one group for steroid analyses and the other group for analysis of gonadotropin binding). Hamsters (8/group) were killed by an overdose of ether at 0900–1000 hr on Day 4 (proestrous controls—0-day delay group), Day 5 (1-day delay group), Day 6 (2-day delay group), and Day 7 (3-day delay group).

For the determination of LH and FSH receptors in whole follicles, the ovaries were removed and placed in ice cold Medium-199 (GIBCO, Grand Island, Nebr.). Each ovary was then dissected from its bursa and the large preovulatory follicles were removed with extra fine needle-tip jewelers forceps. Interstitial tissue and other contaminating tissues were removed from the follicles with forceps and scalpel blade. Then whole follicles (5–13) were placed in 0.6 ml receptor buffer (RB, 0.05 *M* Tris–HCl containing 0.01 *M* $CaCl_2$ and 0.075 *M* $MgCl_2$, pH 7.2 (21)), homogenized by an 8-sec burst of a Tissumizer (No. SDT1810, Tekmar, Cincinatti, Ohio) at high speed at 4°C, and stored frozen until assayed for receptors.

For the assay of steroids in follicular fluids, 10 follicles per animal (eight hamsters/group) were dissected and ruptured into 1.0 ml of 0.9% physiologic saline. Then, the saline containing follicular fluids and granulosa cells was centrifuged for 5 min at 500*g*. The supernatant was aspirated and frozen until assayed for progesterone, androstenedione, and estradiol. The steroid levels in follicular fluid will be expressed as picograms per follicle.

Steroid radioimmunoassay. Concentrations of progesterone, androstenedione, and estradiol were determined in follicular fluid by radioimmunoassay after extraction with anhydrous diethyl ether as described earlier (4).

The antisera and characteristics of the assays have been described previously (4). The intra- and interassay coefficients of variation were less than 10.3% for all assays.

Receptor assays. Binding of iodinated ovine FSH and hCG to follicular homogenates was tested *in vitro*. A sample (50 μl) of follicular homogenate was placed in a 10 × 75-mm disposable glass culture tube, followed by the addition of 25 μl (≃10,000 cpm) ^{125}I-labeled ovine FSH (I-1, National Hormone and Pituitary Agency) or ^{125}I-labeled hCG (CR-119, National Hormone and Pituitary Agency) in receptor buffer containing 0.1% BSA. In addition, 25 μl RB-BSA with and without excess (≃1 μg) gonadotrophin was added to nonspecific binding and the total binding tubes, respectively. The final incubation volume was 0.1 ml. The tubes were then capped and incubated overnight with gentle agitation at room temperature (≃22°C) for 18 hr. The next morning 1 ml RB-BSA was added to each tube and then the tubes were spun at 1500*g* for 30 min at room temperature. The supernatant fractions were aspirated and the pellets counted for radioactivity. Specific total binding was assessed by subtraction of nonspecific binding from total binding; specific binding was expressed as cpm/follicle. Each receptor assay was performed once thus all samples were assayed within the same assay.

Iodination of gonadotrophins. Radioiodination was performed using lactoperoxidase (7). The specific activities of the ^{125}I ovine FSH and hCG were ≃9 and 16 μCi/μg hormone, respectively. Routinely the active fractions of the iodinated hormones were determined immediately after iodination by incubating increasing quantities of homogenized tissues (fixed volume) with a constant amount of radiolabeled gonadotrophin under normal assay conditions. The active fractions for ovine FSH and hCG were ≃25 and 60%, respectively. This represents the maximum amount of binding of the

radiolabeled hormone in the presence of excess receptors.

Statistics. Results were analyzed by analysis of variance (ANOVA) with a one-way design and when appropriate (if ANOVA $P < 0.05$) by Duncan's multiple range test. Differences were considered significant if $P < 0.05$.

Results. *Follicular fluid (Table I).* On Days 1 and 2 of delay, progesterone concentrations in follicular fluid were higher ($P < 0.01$) than that observed on proestrus (Day 0 delay). However, by Day 3 of delay, progesterone concentrations in follicular fluid declined to values similar to those on proestrus. One day of ovulatory delay increased ($P < 0.01$) the concentration of estradiol in follicular fluid and this was followed by a decline ($P < 0.01$) on Days 2 and 3 of delay. As revealed by ANOVA, ovulatory delay did not significantly alter the concentration of androstenedione in follicular fluid; although there was a gradual decline in androstenedione values throughout delay.

FSH AND hCG binding (Table II). Two and three days of ovulatory delay decreased ($P < 0.05$ and 0.01, respectively) the FSH binding to preovulatory follicles. ANOVA revealed no overall effect ($P < 0.06$) of ovulatory delay on hCG binding, even though hCG binding to preovulatory follicles after 2 and 3 days of delay tended to be higher than that on proestrus (0 day).

Discussion. Ovulatory delay in the hamster increased the follicular fluid level of progesterone after 1 day of ovulatory delay. The progesterone in the follicular fluid remained elevated on Day 2 of delay and then declined to control values on Day 3. Previous studies from our lab using hamsters (4, 6) have shown that after 2 and 3 days of delay progesterone production increased in response to LH *in vitro.* Thus the increase in follicular fluid progesterone (Table I) preceded by 1 day the increase in progesterone production *in vitro* (4, 6). This indicates that the level of follicular fluid progesterone relative to that on proestrus may be an indicator of the follicles ability to produce progesterone. Whether the major source of progesterone in the follicular fluid is from theca as has been shown for the *in vitro* production of progesterone by delayed follicles (6) is unknown. The observation that theca promotes granulosa production of progesterone (8) indicates that follicular fluid progesterone might be primarily of granulosal origin. Two lines of evidence are worthy of discussion in determining whether certain steroids are of thecal or granulosal origin. First, theca is the primary source of androstenedione in the hamster follicle (6, 9) and androstenedione production supersedes production of progesterone on proestrus (6), yet the absolute level of androstenedione in follicular fluid is less than that

TABLE I. CONCENTRATIONS OF PROGESTERONE, ANDROSTENEDIONE, AND ESTRADIOL IN FOLLICULAR FLUID OF PREOVULATORY FOLLICLES ON PROESTRUS AND AFTER 1, 2, AND 3 DAYS OF OVULATORY DELAY

	Steroid (pg/follicle)		
Treatment group	Progesterone	Androstenedione	Estradiol
Proestrous follicles			
0 day	36.7 ± 10.5	8.6 ± 1.3	72.8 ± 13.7
Delayed follicles			
1 day	105.6 ± 12.3*	7.1 ± 0.6	149.8 ± 5.2*
2 day	131.8 ± 22.7**	6.1 ± 1.4	47.4 ± 8.0**,***
3 day	84.6 ± 25.1	4.9 ± 0.6	43.7 ± 16.9***
One-way ANOVA:			
Δ(P value)	(0.01)	(0.14)	(0.0001)

Note. Values are means ± SEM for $n = 8$ hamsters per group and the follicular fluid from 10 follicles per hamster were pooled for a single determination.

*,** $P < 0.05$ or $P < 0.01$ compared with proestrous value, respectively, by Duncan's test.

*** $P < 0.01$ compared with 1 day value by Duncan's test.

TABLE II. FSH AND hCG BINDING OF PREOVULATORY FOLLICLES ON PROESTRUS AND AFTER 1, 2, OR 3 DAYS OF OVULATORY DELAY

	cpm/follicle	
Treatment group	FSH	hCG
Proestrous follicles		
0 day	3772 ± 362	1626 ± 398
Delayed follicles		
1 day	2980 ± 464	1722 ± 809
2 day	2319 ± 414*	4171 ± 1007
3 day	1274 ± 279**	2456 ± 726
1-way ANOVA:		
Δ(*P* value)	(0.0007)	(0.06)

Note. Values are mean ± SEM for $n = 8$ hamsters per group; ≃10 follicles per animal were used.

*,** $P < 0.05$ and 0.01, respectively, when compared with proestrous values by Duncan's test.

of progesterone. Thus steroids produced by theca may not be principally incorporated into follicular fluid. In the rabbit, only a very small portion of steroids secreted (thecal origin) into the ovarian vein are reflected in follicular fluid levels (10). Interestingly, androstenedione levels in follicular fluid did not decline significantly (Table I) even though a drastic decline in *in vitro* follicular androstenedione production has been observed on Days 2 and 3 of delay (4). Second, the absolute level of estradiol in follicular fluid was at least eight times higher than that of androstenedione; this is apparently due to the granulosa cells being the site of aromatization of androgen to estrogen in the follicle (11) and due to the granulosa cell secretion of estrogen directly into the follicular fluid.

Another explanation for the low level of androstenedione in follicular fluid is that the androstenedione is aromatized very quickly by the granulosa thus leaving little androstenedione for accumulation in the follicular fluid. It is well established that the aromatizing activity of the granulosa cells in hamsters is very high (11).

The abrupt increase in the follicular fluid level of estradiol after 1 day of delay is interesting and remains unexplained. A previous study has shown an increase in aromatizing capacity of 1- and 2-day delayed follicles and granulosa cells (4, 6). The decline in the follicular fluid level of estradiol on Days 2 and 3 parallels somewhat the decline in LH-stimulated estradiol production of delayed follicles on those days (4). In the rat, an increase in follicular estradiol has been observed after 2 days of delay (5).

Ovulatory delay in the hamster was associated with a significant decrease in FSH binding to homogenates of whole preovulatory follicles (Table II). Thus, the relative ratio of hCG:FSH binding favors hCG. A previous report using the pentobarbital-treated rat has shown that after 1 day of ovulatory delay hCG binding was higher than in nondelayed proestrus controls (2); thereafter hCG binding decreased. FSH binding to preovulatory follicles of the rat steadily declined during barbituate-induced ovulatory delay (2). Using anti-PMSG to induce atresia of preovulatory follicles in the PMSG-treated hypophysectomized hamster, FSH binding as determined by autoradiography declined abruptly and hCG binding declined more slowly (12). When compared with the barbiturate model, the changes in hCG and FSH binding occurred at a faster rate in the PMSG–anti-PMSG model because of the acute neutralization of PMSG. In these models, the alterations in hCG and FSH binding tend to favor a higher hCG:FSH binding ratio.

The increase in hCG:FSH binding ratio correlates well with the increase in *in vivo* follicular fluid progesterone in the present study and the *in vitro* progesterone production previously reported (1, 2, 4). Previous studies have shown a reduced binding of FSH and an increase in hCG binding to corpora lutea (13) at a time when large amounts of progesterone are contained within and being produced by the corpora lutea (14). The delayed follicles might become very sensitive to the circulating LH. This increase in sensitivity to LH may mimic the previously reported inhibitory action of LH on follicular androgen and estrogen production (15, 16) and thus, might be causally related to the induction of atresia. The transient increase in sensitivity of the delayed follicles to LH might be the "trigger" that leads to atresia, the first signs being a decrease in androgen and estrogen bisynthesis. Indeed, the 17α-hydroxylase and C17-20 lyase activities are reduced in delayed follicles (17).

Various models studying atresia of preovulatory follicles in rats and hamsters have shown a transient increase in *in vitro* and *in vivo* progesterone and a decline in androgen and estrogen production (1, 2, 4, 18–20). Thus, the latter changes in steroidogenesis appear to be the result of ovulatory delay and atresia and due to neither the "tools" (barbiturate, anti-PMS) used to induce atresia nor the species.

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