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An Alteration in the Hypothalamic Action of Estradiol Due to Lack of Progesterone Exposure Can Cause Follicular Cysts in Cattle[1]

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ABSTRACT

Many mammals, including cattle, can develop ovarian follicular cysts, but the physiological mechanisms leading to this condition remain undefined. We hypothesized that follicular cysts can develop because estradiol will induce a GnRH/LH surge on one occasion but progesterone exposure is required before another GnRH/LH surge can be induced by estradiol. In experiment 1, 14 cows were synchronized with an intravaginal progesterone insert (IPI) for 7 days, and prostaglandin $F_{2\alpha}$ was given on the day of IPI removal. Estradiol benzoate (EB; 5 mg i.m.) was given 3 days before IPI removal to induce atresia of follicles. Cows were given a second EB treatment 1 day after IPI removal to induce a GnRH/LH surge in the absence of an ovulatory follicle. All cows had an LH surge following the second EB treatment, and 10 of 14 cows developed a large-follicle anovulatory condition (LFAC) that resembled follicular cysts. These LFAC cows were given a third EB treatment 15 days later, and none of the cows had an LH surge or ovulation. Cows were then either not treated (control, n = 5) or treated for 7 days with an IPI (n = 5) starting 7 days after the third EB injection. Cows were treated for a fourth time with 5 mg of EB 12 h after IPI removal. All IPI-treated, but no control, cows had an LH surge and ovulated in response to the estradiol challenge. In experiment 2, cows were induced to LFAC as in experiment 1 and were then randomly assigned to one of four treatments 1) IPI + EB, 2) IPI + GnRH (100 μg), 3) control + EB, and 4) control + GnRH. Control and IPI-treated cows had a similar LH surge and ovulation when treated with GnRH. In contrast, only IPI-treated cows had an LH surge following EB treatment. Thus, an initial GnRH/LH surge can be induced with high estradiol, but estradiol induction of a subsequent GnRH/LH surge requires exposure to progesterone. This effect is mediated by the hypothalamus, as evidenced by similar LH release in response to exogenous GnRH. This may represent the physiological condition that underlies ovarian follicular cysts.

estradiol, follicle, hypothalamus, ovary, progesterone

INTRODUCTION

Ovarian follicular cysts have been reported in many mammalian species. In cattle, this condition is characterized by large (generally ≥25 mm in diameter) anovulatory structures on the ovary in the absence of a corpus luteum (CL). In dairy cattle, this condition has been estimated to occur in 6%–19% of animals and is reported to be an important cause of infertility [1, 2]. An understanding of the underlying physiological cause of ovarian follicular cysts in cattle may provide insight regarding this condition in other species, including humans.

Ultrasound evaluation of the ovaries clearly shows that follicular cysts in cattle are not static structures but, rather, that waves of follicular growth occur, even in the presence of large anovulatory cysts [3]. During the 1970s, researchers found that multiple treatments with high doses of progesterone and estradiol would induce follicular cysts [4]. It is now common to induce ovarian follicular cysts with this protocol [3, 5, 6], although only approximately half of treated cows develop follicular cysts.

To our knowledge, the underlying mechanisms that produce follicular cysts following this treatment have not been defined. We hypothesized that the physiological condition underlying follicular cysts relates to a hypothalamic lesion such that estradiol cannot induce a GnRH/LH surge. This idea is consistent with the previous work of Dobson and Alam [7]. Furthermore, we hypothesized that this condition is established by estradiol inducing a GnRH/LH surge that is not followed by an increase in circulating progesterone. To test this hypothesis, we attempted to design an animal model in which we could cause an estradiol-induced GnRH/LH surge in the absence of an ovulatory follicle. We eliminated a potentially ovulatory follicle by treatment of cows with estradiol in the presence of high progesterone concentrations. Previous researchers have shown that this treatment will decrease FSH concentrations and cause atresia of any dominant follicles [8, 9]. Emergence of a new follicular wave occurred approximately 4.3 ± 0.1 (mean ± SEM) day after the estradiol treatment. We used this animal model to test whether treatment with a single estradiol injection would induce a large-follicle anovulatory condition (LFAC), similar to ovarian follicular cysts, if the cow did not ovulate following an estradiol-induced GnRH/LH surge. We further tested whether the lack of an estradiol-induced GnRH/LH surge was due to a lesion at the hypothalamus or pituitary and whether progesterone exposure could eliminate this lesion.

MATERIALS AND METHODS

General Animal Procedures

Two experiments were conducted at the University of Wisconsin-Madison Dairy Research Center between 2 August and 9 December 1999. Thirty-four Holstein, nonlactating, nonpregnant dairy cows were used in these two experiments. Cows were housed outside in an open lot except during bleeding or ultrasound scanning, when they were moved to a stanchion barn. They were fed the refusals from a dairy cow ration (feed that was not consumed by dairy cows at 24 h after feeding) supplemented with alfalfa hay as needed. All animal handling and care procedures were approved by the Research Animal Resources Center of University of Wisconsin-Madison.

Ovarian ultrasonographic examinations were performed as previously described [10] using a real-time, B-mode scanner equipped with a 7.5-MHz, linear-array, intrarectal transducer (Aloka 500V; Corometrics Medical Systems, Inc., Wallingford, CT). Measurements were made on a single

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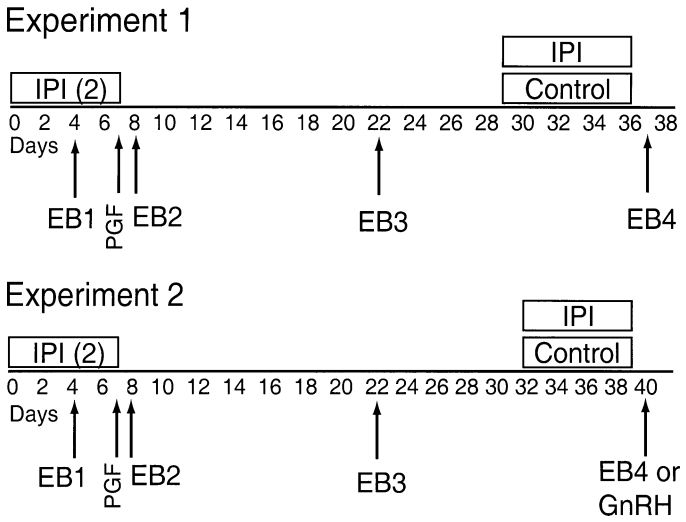

FIG. 1. Summary of experimental protocols showing treatments to induce the anovulatory condition and subsequent treatment with IPI. Two IPIs were inserted to increase serum progesterone concentration, and an initial injection of EB (EB1; 5 mg i.m.) was given to eliminate all potentially ovulatory follicles. PGF (500 μg i.m.) was given to regress any luteal tissue. At 1 day after PGF, a second EB injection (EB2; 5 mg i.m.) was given to induce GnRH/LH surge in the absence of an ovulatory follicle. Subsequent challenge was also made with a third EB injection (EB3; 5 mg i.m.) to determine if cows were refractory to estradiol. All cows were challenged with a fourth EB injection (EB4; 5 mg i.m.) in experiment 1 and with EB4 (5 mg i.m.) or GnRH (100 μg i.m.) in experiment 2.

frozen image of the apparent maximal area of each follicle using the average diameter in two directions at right angles. Transrectal ultrasound scanning to monitor follicles was performed every day (experiment 1) or every other day (experiment 2) beginning on the day of administration of the progesterone inserts (Day 0). Ovulation was determined by ultrasound scanning (i.e., disappearance of a large follicle followed by appearance of a corresponding CL) and confirmed by an increase in serum progesterone concentration.

Chemicals

Prostaglandin $F_{2\alpha}$ (PGF) analogue (Estrumate) was from Bayer Corporation (Shawnee Mission, KS). Sesame oil and β-estradiol 3-benzoate were from Sigma Chemical Co. (St. Louis, MO). Benzyl alcohol was from EM Science (Cherry Hill, NJ). The intravaginal progesterone inserts (IPIs; Eazi-Breed CIDR containing 1.9 g of progesterone) were from InterAg Company (Hamilton, New Zealand). Cystorelin was from Merial, Inc. (Iselin, NJ).

Blood Sampling

Blood samples for progesterone analysis were collected from the coccygeal vein into Vacutainer tubes (Becton Dickinson and Co., Franklin Lakes, NJ) every other day from Day 0 until Day 48 in experiment 1 and until Day 50 in experiment 2. In experiment 1, blood samples were also collected for LH determination at 0, 6, 12, 14, 16, 18, 20, 22, 24, and 30 h after each estradiol benzoate (EB) injection. In experiment 2, blood samples for LH were collected at 0, 12, 14, 16, 18, 20, 22, and 24 h after the third and fourth EB injection or every 30 min from 0 to 4 h after GnRH injection. Blood was allowed to clot at 4°C for 24 h and then centrifuged at 3000 rpm for 15 min. Serum was poured into sample tubes and stored at −20°C until assays were performed.

Hormone Assays

Serum concentration of LH was determined using an RIA validated for use in cattle [11, 12]. The LH assay incorporated USDA-bLH-B-6 for iodination and reference standards and USDA-309-684p as the primary antiserum. Hormone sensitivity, calculated as two SDs below the mean cpm at maximum binding, was 0.06 ng/ml. Coefficients of variation for within and between assays for LH were 5.4% and 6.3%, respectively, using pooled plasma from cows near estrus (mean, 2.1 ng/ml of LH).

Serum concentration of progesterone was determined by an ELISA as described previously [13]. Coefficients of variation for within and between assays for progesterone were 5.2% and 8.0%, respectively, using a quality-control sample with 2.5 ng/ml of progesterone.

Experiment 1

The protocol for experiment 1 is summarized in Figure 1. Cows at unknown stages of the estrous cycle (n = 14) were treated with two IPIs for 1 wk (Days 0–7). Injection of EB (5 mg i.m.) was administrated 4 days after IPI insertion to induce atresia of any dominant follicles. The IPIs were removed and PGF (500 μg i.m.) administrated at Day 7 to regress any CL. A second treatment with EB (5 mg i.m.) was given to cause an LH surge 1 day after the PGF injection and IPI removal. Ovaries of all cows were evaluated daily for the next 14 days, and a third EB treatment (5 mg i.m.) was then given to cows. Cows that ovulated or luteinized follicles were removed from the experiment. The above treatments were designed to induce an anovulatory condition (first and second EB treatments) or to determine the estradiol-responsiveness of cows in the anovulatory condition (third EB treatment). At 1 wk after the third EB treatment (Day 29), anovulatory cows were assigned into two groups: IPI-treated cows, or untreated controls. The IPI group was treated for 1 wk with a single IPI (Days 29–36). All cows were given a fourth EB treatment (5 mg i.m.) 12 h after IPI removal.

Experiment 2

The protocol for experiment 2 is summarized in Figure 1. Twenty cows were used in this experiment. The same protocol was used to induce anovulation as in experiment 1. Cows were randomly assigned at Day 32 (after IPI insertion) to one of four treatment groups: 1) IPI + EB, 2) IPI + GnRH, 3) control + EB, or 4) control + GnRH. Cows in the IPI groups were given an IPI for 1 wk from Day 32 through Day 39. Control cows were left untreated during this same time period. Cows were administered either EB (5 mg i.m.) or GnRH (100 μg i.m.) 12 h after IPI removal.

Statistical Analysis

Measurements obtained before treatments were used as covariates for statistical analyses of corresponding hormone concentrations. Analysis of covariance was conducted using the proc mixed procedure of the Statistical Analysis System [14] with repeated measures in time as a subplot to test the effect of treatments on circulating hormones. Cow nested within treatments and group of cows was the random error term for all data analyses. Probability of significance was generated using the Satterthwaite approximation [15], because the number of cows in the experimental groups was unequal. Probability values of less than or equal to 0.05 were considered to be significant in both experiments.

RESULTS

Experiment 1

Ten of 14 (71%) cows were induced into LFAC that resembled follicular cysts. Dominant follicles regressed after the first EB injection, and a new follicular wave began in all cows except one, which did not regress the dominant follicle after the first EB injection and, subsequently, ovulated this follicle after the second EB injection. Another 3 cows were removed from the experiment, because they spontaneously ovulated between the second and third EB injections. As illustrated by the cow shown in Figure 2, follicular waves were observed throughout the experimental period. Most of the cows (7 of 10) grew follicles to a maximum diameter of approximately 20 mm. The other 3 cows grew follicles to greater than 25 mm in diameter.

As expected, none of the cows had an LH surge after the first EB injection due to the elevated circulating progesterone concentrations present at the time of injection (Fig. 3A). All cows had an LH surge following the second EB injection (Fig. 3B). The anovulatory cows (n = 10) were challenged with a third EB injection (5 mg i.m.), and an LH surge was not detectable in any of the cows (Fig. 3C). All progesterone-treated cows showed an LH surge

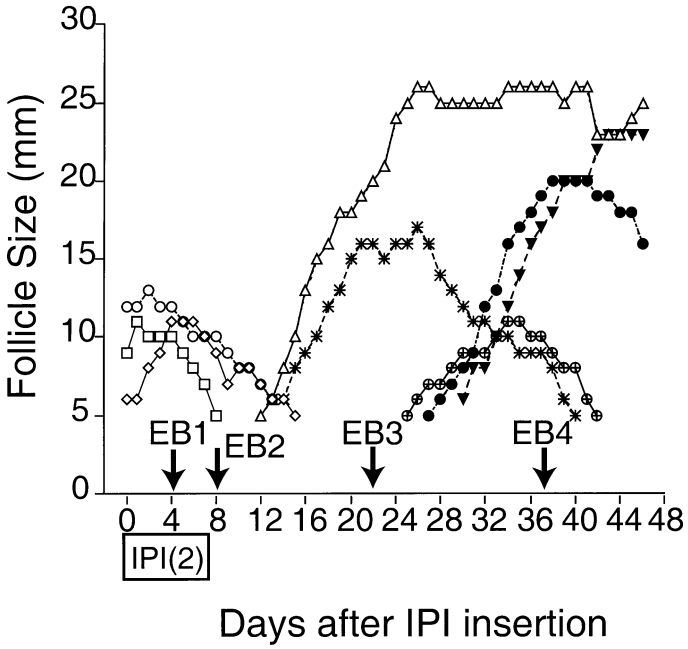

FIG. 2. Growth pattern of dominant follicles after insertion of two IPIs in one anovulatory control cow in experiment 1. The EB treatments (5 mg i.m.) are shown with arrows.

and ovulation following the fourth EB injection (5 mg i.m.). Three of 5 cows ovulated 2 follicles following this injection, whereas the other 2 cows each had a single ovulation. No LH surge or ovulation was observed in the control cows following the fourth EB injection (5 mg i.m.) (Fig. 3D).

Progesterone concentration was similar between ovulatory and anovulatory cows until Day 14. Subsequently, ovulatory cows had an increased serum progesterone concentration (Fig. 4) and a new, growing CL. All anovulatory cows had a low progesterone concentration until IPI insertion (in the IPI-treated cows). The IPI-treated cows showed an increased serum progesterone concentration, to approximately 1 ng/ml, due to insertion of IPIs. A much greater increase in serum progesterone concentrations in IPI-treated cows was observed following the fourth EB injection due to the induced ovulation. Control cows had low circulating progesterone concentrations throughout the experimental period after PGF treatment (Fig. 5).

Experiment 2

Ten of 20 cows (50%) were induced into LFAC. Dominant follicles regressed in most cows (n = 17) after the first EB administration (5 mg i.m.). The dominant follicles did not regress in 3 cows in response to the first EB injection, and these cows ovulated after the second EB injection (5 mg i.m.). Two cows spontaneously ovulated between the second and third (5 mg i.m.) EB treatments, and 5 cows ovulated after the third EB injection. The remaining anovulatory cows (n = 10) demonstrated follicular waves throughout the experimental period, as shown for one representative cow in Figure 6.

Anovulatory cows did not show an LH surge after the third EB injection (5 mg i.m.). All of the IPI-treated cows, but none of the control cows, had an LH surge and ovulation after the fourth EB injection (5 mg i.m.). Treatment with 100 μg of GnRH caused an LH surge of similar magnitude and ovulation in both progesterone-treated and control cows (Fig. 7).

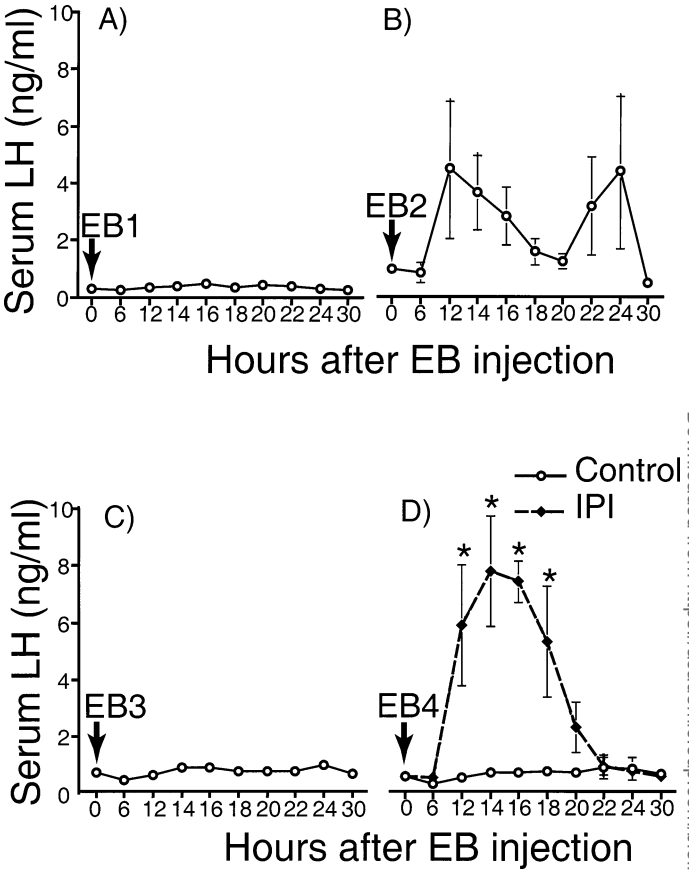

FIG. 3. Serum LH concentration (mean ± SEM) following EB treatment (5 mg i.m.) in experiment 1. **A**) The first EB injection (EB1) was given to regress functional follicles during high progesterone concentration supplied by IPIs for 7 days (Days 0–7). **B**) The second EB injection (EB2) was given to cause a GnRH/LH surge in the absence of ovulatory follicles. **C**) The third EB injection (EB3) was given to challenge the anovulatory cows (n = 10) to assure a lack of GnRH/LH surge after elevated estradiol. **D**) The fourth EB injection (EB4) was given to determine responsiveness to estradiol following IPI treatment. *Significant difference ($P < 0.001$) between progesterone-treated vs. control cows.

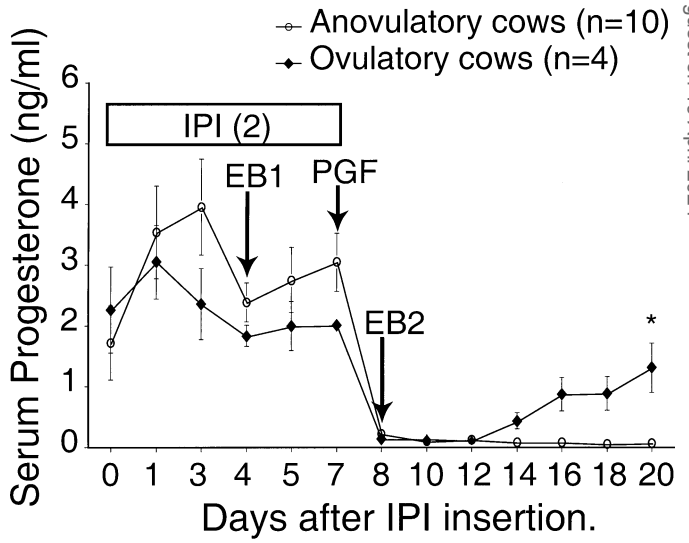

FIG. 4. Serum progesterone concentration (mean ± SEM) after insertion of two IPIs in experiment 1. The EB (5 mg i.m.) and PGF (500 μg i.m.) treatments are shown with arrows. *Significant difference ($P < 0.05$) between ovulatory vs. anovulatory cows.

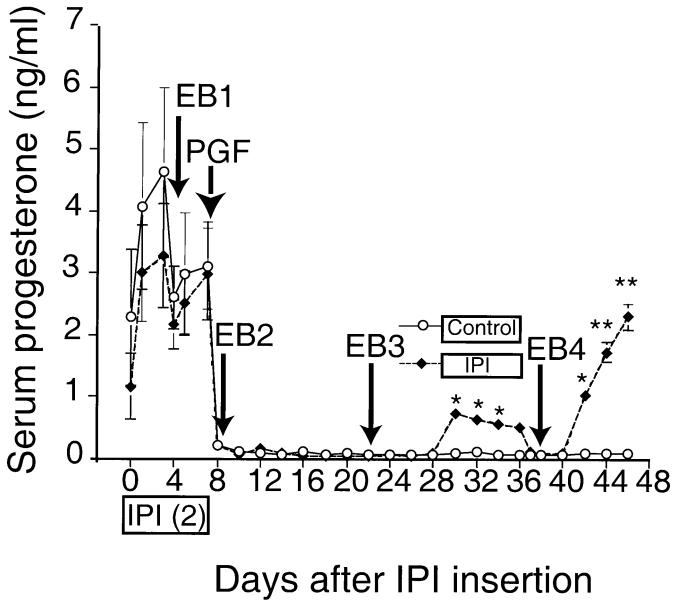

FIG. 5. Serum progesterone concentration (mean ± SEM) in anovulatory cows after insertion of IPIs in experiment 1. The EB (5 mg i.m.) and PGF (500 μg i.m.) treatments are shown with arrows. IPI was given to elevate serum progesterone concentration between Days 29 and 36 in IPI-treated cows (n = 5). Untreated cows served as controls (n = 5). $^{*}P < 0.05$ and $^{**}P < 0.001$ between progesterone-treated vs. control group.

Serum progesterone concentration was similar in all anovulatory cows until IPI treatment. The IPI-treated cows had elevated progesterone concentrations (~1 ng/ml) during IPI treatment. Three of the groups of cows (IPI + EB, IPI + GnRH, and control + GnRH) had an increasing progesterone concentration following treatment due to ovulation and subsequent growth of a CL. Cows in the control + EB group had low progesterone concentrations throughout the experiment after PGF treatment (Fig. 8).

DISCUSSION

Many species have anovulatory states that are characterized by the growth and persistence of follicles larger than

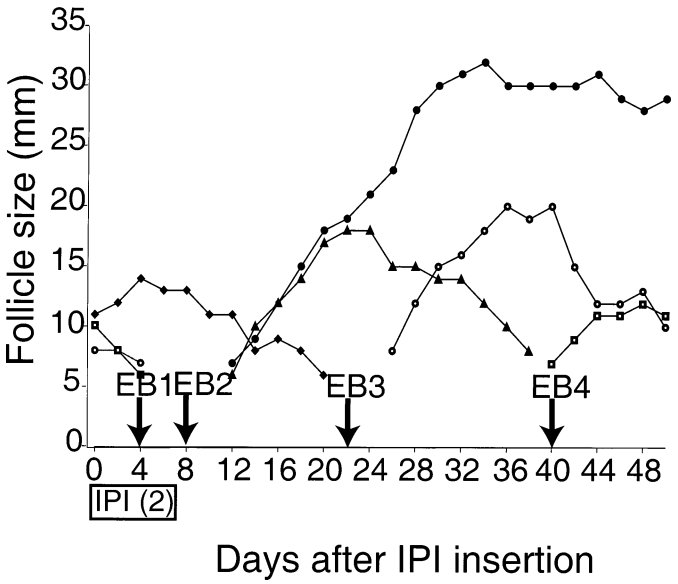

FIG. 6. Growth pattern of dominant follicles for one anovulatory control cow in experiment 2. Two IPIs were inserted between Days 0–7. The EB treatments (5 mg i.m.) are shown with arrows.

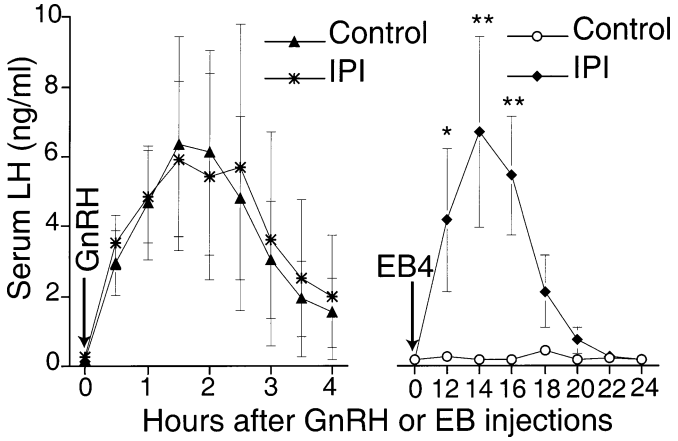

FIG. 7. Serum LH concentration (mean ± SEM) after either GnRH (100 μg i.m.) or EB (5 mg i.m.) treatments in experiment 2. The IPI group was treated with an IPI for 7 days, and both injections (GnRH or EB) were given 12 h after IPI removal. $^{*}P < 0.05$ and $^{**}P < 0.001$ between progesterone-treated vs. control group.

the normal ovulatory size. For example, in aged rats, large estrogen-active follicles spontaneously grow on the ovaries without subsequent LH surges or ovulation [16, 17]. These large anovulatory follicles have generally been termed follicular cysts and have been described in rats, rabbits, swine, dogs, sheep, cattle, and women [1, 16, 18–23]. The diameter of a normal ovulatory follicle in dairy cattle is 16 ± 0.4 or 13.9 ± 0.4 mm (2 and 3 follicular waves, respectively, during an estrous cycle) [24]. In the present study, we described an anovulatory state in cows during which follicles grew to greater than ovulatory size and, sometimes, greater than the size that has classically been defined as follicular cysts (diameter, >25 mm), but these follicles did not proceed to ovulation. Our results are consistent with the underlying physiological lesion in this large-follicle anovulatory state being the lack of an estradiol-induced

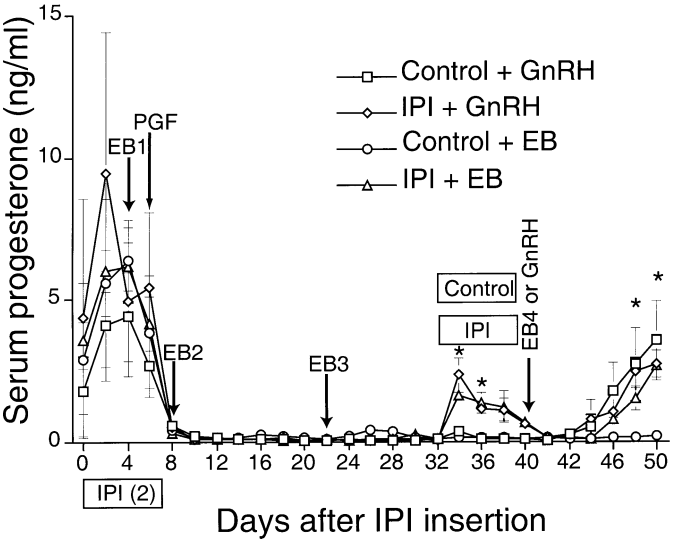

FIG. 8. Serum progesterone concentration (mean ± SEM) after insertion (Days 0–7) of IPI in anovulatory cows in experiment 2. The EB (5 mg i.m.) and PGF (500 μg i.m.) treatments are shown with arrows. IPI was given to elevate serum progesterone concentration between Days 32 and 39 in IPI-treated cows, and the fourth EB injection (EB4; 5 mg i.m.) or GnRH (100 μg i.m.) was given 12 h after IPI removal. $^{*}P < 0.05$ between control + EB vs. the other treatment groups.

GnRH/LH surge due to a hypothalamic insensitivity to estradiol.

Researchers have reported many methods for induction of LFAC, including ACTH [25, 26], endotoxin [27, 28], overfeeding [20, 29], and induction of hypothyroidism [30]. Treatment with high levels of estradiol for prolonged time periods has been a classical method to produce this condition in cattle [3–6] and rats [16]. More recent, elegant studies in ovariectomized sheep [31] have characterized the time requirement for estradiol exposure and found that treatment for 2–12 days with follicular-phase levels of estradiol blocked a subsequent estradiol-induced LH surge in most ewes. The positive feedback effects of estradiol returned after progesterone treatment [31]. The present study used a relatively simple induction protocol based on the hypothesis that progesterone is required to reset the estradiol/GnRH/LH surge mechanism following an estradiol-induced GnRH/LH surge. Previous researchers have reported that cattle with follicular cysts did not have an LH surge in response to exogenous estradiol [7, 32, 33]. In addition, it has been reported that progesterone [34–36] and GnRH [37–39] are an effective treatment for follicular cysts. In the present study, induction of a single GnRH/LH surge was consistently followed by an LFAC if the LH surge was not followed by an increase in circulating progesterone concentrations.

The LH surge in ruminants is clearly due to a surge in GnRH in response to elevated circulating estradiol concentrations [40]. Estradiol appears to act on cells in the mediobasal hypothalamus [41, 42] by activating estrogen receptor (ER) α [43]. Several studies have shown that ERα knockout mice have very large anovulatory follicles [44–47]. This seems to be logical, because the positive feedback action of estradiol in inducing a GnRH/LH surge appears to be mediated through hypothalamic ERα [48]. Of particular importance to the present study, it has also been shown that progesterone-receptor knockout (PRKO) mice are anovulatory [49, 50]. The PRKO mice have follicles of greater than ovulatory size [49] and normal levels of circulating estradiol [50], but they do not exhibit ovulation or luteinization. The lack of ovulation is due to both an intraovarian problem (hCG treatment could not induce ovulation) and a hypothalamic defect (estradiol did not induce preovulatory LH or FSH surges) [50].

The hypothalamic changes that produce estradiol insensitivity in our studies are unclear, but they may involve reduced ER in critical hypothalamic regions. In rats, treatment with estradiol specifically downregulates hypothalamic ER mRNA in the female [51, 52] but not in the male [53]. Treatment with progesterone could subsequently increase ER mRNA [54]. In ovariectomized ewes, Blache et al. [55] used immunohistochemistry to localize hypothalamic ER after treatment with various steroids. Progesterone and estradiol treatment did not alter the density of positive-staining cells in the medial preoptic area or arcuate nucleus, but density and distribution were dramatically altered in the mediobasal hypothalamus after hormonal treatments. Progesterone treatment specifically increased ER in the lateral part of the mediobasal hypothalamus, the region that was previously found to be most sensitive to the effect of estradiol microimplants on the LH surge [41, 56].

A relatively simple model could explain our results based on these previous morphological and physiological studies (Fig. 9). An estradiol surge, either due to the final stages of preovulatory follicular growth or in response to exogenous estradiol treatment, could not only induce a

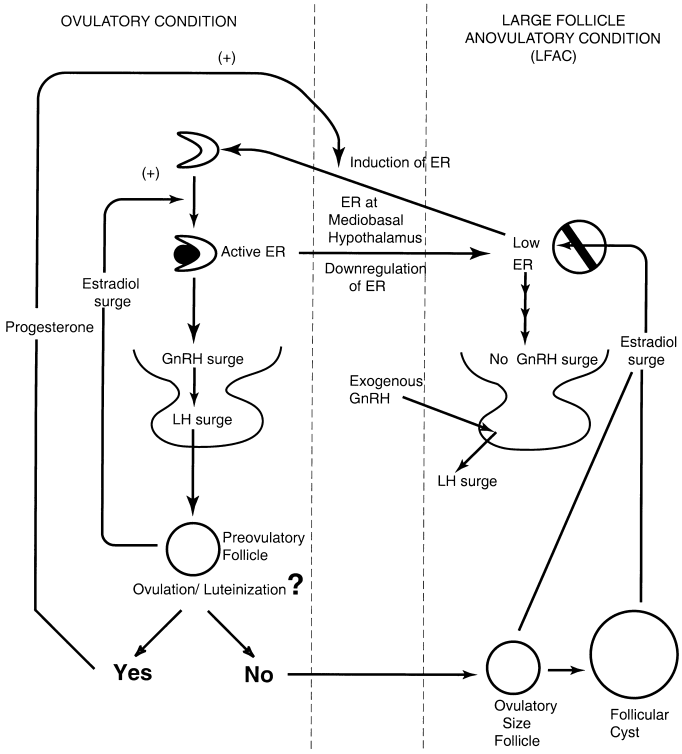

FIG. 9. Simplified model of possible underlying physiology that could explain LFAC and results from the present study. The estradiol surge strongly activates ERs in the mediobasal hypothalamus, causing a GnRH/LH surge and downregulation of ER. If no ovulation or subsequent increase in progesterone occurs after the GnRH/LH surge, the animal enters a state of refractoriness to the positive feedback effects of estradiol. A subsequent increase in progesterone due to ovulation/luteinization or progesterone treatments causes induction of ER that reintroduces hypothalamic responsiveness to an estradiol surge. Even in anovulatory animals, GnRH can cause LH release from pituitary.

GnRH surge but also downregulate expression of ERα in the lateral mediobasal hypothalamus. A deficiency in functional ERα in this critical region could prevent subsequent induction of a GnRH surge in response to estradiol. Progesterone may induce ERα in the mediobasal hypothalamus. Thus, progesterone treatment in our experiments, or in previous experiments, could reinitiate responsiveness to estradiol by simply increasing functional ERα in a region of the hypothalamus that is critical for inducing the GnRH surge. Certainly, much more complicated cellular models could also explain our results. Regardless of the precise cellular mechanisms, it seems to be clear that the anovulatory condition induced in our experiments is caused by a hypothalamic and not by a pituitary lesion, as evidenced by the normal response to GnRH. Under natural conditions, this could occur if a GnRH surge were induced but then not followed by ovulation. For example, a GnRH surge could be induced in animals that have reduced pituitary LH, resulting in an LH surge that is not adequate to induce ovulation/luteinization, with no subsequent CL development or rise in circulating progesterone. Alternatively, a follicular defect could prevent ovulation in response to a GnRH/LH surge, and a subsequent lack of progesterone could produce this anovulatory condition. Other scenarios involving the hypothalamus, pituitary, or ovary could also be envisioned that may lead to a GnRH surge without a subsequent increase in progesterone. Further research is warranted to better understand this intriguing anovulatory

condition characterized by large follicles in the absence of an estradiol-induced GnRH/LH surge.

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