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Gonadotropin-Releasing Hormone, Estradiol, and Inhibin Regulation of Follicle-Stimulating Hormone and Luteinizing Hormone Surges: Implications for Follicle Emergence and Selection in Heifers[1]

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ABSTRACT

Mechanisms regulating gonadotropin surges and gonadotropin requirements for follicle emergence and selection were studied in heifers. Experiment 1 evaluated whether follicular inhibins regulate the preovulatory luteinizing hormone (LH)/follicle-stimulating hormone (FSH) surges elicited by gonadotropin-releasing hormone (GnRH) injection (Hour = 0) and the subsequent periovulatory FSH surge. Treatments included control (n = 6), steroid-depleted bovine follicular fluid (bFF) at Hour −4 (n = 6), and bFF at Hour 6 (n = 6). Gonadotropins in blood were assessed hourly from Hours −6 to 36, and follicle growth tracked by ultrasound. Consistent with inhibin independence, bFF at Hour −4 did not impact the GnRH-induced preovulatory FSH surge, whereas treatment at Hour 6 delayed onset of the periovulatory FSH surge and impeded growth of a new follicular wave. Experiment 2 examined GnRH and estradiol (E2) regulation of the periovulatory FSH surge. Treatment groups were control (n = 8), GnRH-receptor antagonist (GnRHr-ant, n = 8), and E2 + GnRHr-ant (n = 4). GnRHr-ant (acyline) did not reduce the concentrations of FSH during the periovulatory surge and early follicle development (<7.0 mm) was unaffected, although subsequent growth of a dominant follicle (>8.0 mm) was prevented by GnRHr-ant. Addition of E2 delayed both the onset of the periovulatory FSH surge and emergence of a follicular wave. Failure to select a dominant follicle in the GnRHr-ant group was associated with reduced concentrations of LH but not FSH. Maximum diameter of F1 in controls (13.3 ± 0.5 mm) was greater than in both GnRHr-ant (7.7 ± 0.3 mm) and E2 + GnRHr-ant (6.7 ± 0.8 mm) groups. Results indicated that the periovulatory FSH surge stems from removal of negative stimuli (follicular E2 and inhibin), but is independent of GnRH stimulation. Emergence and early growth of follicles (until about 8 mm) requires the periovulatory FSH surge but not LH pulses. However, follicular deviation and late-stage growth of a single dominant follicle requires GnRH-dependent LH pulses.

estradiol, FSH, follicular development, GnRH, inhibin, LH

INTRODUCTION

Despite recent advances elucidating the molecular and cellular mechanisms regulating gonadotropin secretion and follicular development, the in vivo physiological relationships regulating these processes are not adequately defined. In a number of species, two distinct follicle-stimulating hormone (FSH) surges have been described before and encompassing ovulation (for example, rodent [1], rabbit [2], porcine [3], ovine [4], canine [5], bovine [6]). It is well known that FSH synthesis and secretion can be increased by hypothalamic factors, particularly gonadotropin-releasing hormone (GnRH), and inhibited by the ovarian factors estradiol (E2) and inhibin [7, 8]. However, the interaction of these three factors in the regulation of each of the two FSH surges has not yet been clearly determined in vivo.

The E2-induced preovulatory surge of GnRH stimulates surges of both luteinizing hormone (LH) and FSH in the peripheral circulation [9], suggesting by association that the preovulatory FSH surge, like the LH surge, is GnRH dependent. Concentrations of follicular inhibin, a suppressor of FSH synthesis and secretion, appear to be elevated during and fall shortly after the preovulatory LH and FSH surges [10]. Thus, the preovulatory FSH surge arises despite high concentrations of follicular inhibin within the preovulatory follicle. It remains to be tested whether follicular inhibins negatively regulate these GnRH-dependent FSH surges under normal physiological conditions; however, one study [11] did not observe an effect of inhibin treatment on the GnRH-induced LH and FSH surge in anestrous or chronically ovariectomized ewes. Therefore, in experiment 1 we tested the hypothesis that treatment with exogenous inhibins would not alter the magnitude of the GnRH-dependent, preovulatory FSH or LH surges. This was done by treating follicular-phase heifers with bovine follicular fluid (bFF) just before GnRH treatment to induce preovulatory LH and FSH surges.

The preovulatory LH and FSH surges occur concomitantly and are followed immediately by a periovulatory surge in FSH concentrations [7, 12]. The periovulatory FSH surge is critical for the growth of follicles during the periovulatory follicular wave [13–15]. Characterization of circulating hormone concentrations during the periovulatory period temporally associates the onset of the periovulatory FSH surge with a decline in inhibin, specifically inhibin A [10, 16]. In numerous species, treatment with inhibin delays onset of the periovulatory FSH surge (rodents [17], sheep [18], swine [3], cattle [14]). These studies indicate that reduced inhibin following the preovulatory LH/FSH surges is necessary for production of the periovulatory FSH surge; however, the roles of other known regulators of FSH synthesis and secretion, particularly GnRH and E2, have not been investigated in cattle.

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Regarding the role of E2, a close temporal relationship between declining E2 concentrations and onset of the periovulatory FSH surge [19] provides a temporal indication that the periovulatory decline in E2 is essential to the development of the periovulatory FSH surge. Experiment 2 was designed to verify the importance of the relationship between declining E2 following the preovulatory LH/FSH surges and production of the periovulatory FSH surge. This was done by prolonging preovulatory E2 concentrations throughout the expected onset of the periovulatory FSH surge.

Regarding the role of GnRH, previous experiments have suggested that FSH secretion during the periovulatory FSH surge is not acutely dependent on GnRH stimulation. Elimination of neural input from the mediobasal hypothalamus in rodents [20] or treatment of rabbits with a GnRH-receptor antagonist (GnRHr-ant) [2] did not disrupt the FSH surge that followed the preovulatory LH/FSH surges. Cycling ewes treated with pentobarbitone had suppressed preovulatory LH and FSH surges; however, similar treatment did not affect the periovulatory FSH surge [21]. Although specific information regarding the periovulatory FSH surge was not reported, heifers continuously infused with a GnRH agonist to induce pituitary desensitization maintained elevated FSH concentrations for more than 25 days from onset of agonist treatment [22]. In contrast, Fike et al. [23] observed a reduction in FSH concentrations in heifers treated with a GnRHr-ant 2 days after estrus but not in heifers treated with GnRHr-ant 7 or 12 days after estrus. Clearly there is a need to further clarify how GnRH regulates the periovulatory FSH surge.

Follicular deviation, or the beginning of a rapid change in growth rate between the future dominant and subordinate follicles, is used to indicate that selection of a single dominant follicle has occurred [15]. Declining FSH concentrations following the peak of the periovulatory FSH surge are necessary to initiate follicular deviation. Near the time of follicular deviation there are increases in LH concentrations [24], increased LH receptor expression in granulosa cells [25–28], and increased LH responsiveness [29]. Maximal diameter of the dominant follicle is not attained when LH concentrations are reduced with a GnRHr-ant [23] or by chronic treatment with a GnRH-receptor agonist [22]. The smaller attained diameter of the dominant follicle indicates that the selection mechanism was impaired; however, critical data pertaining to growth patterns and deviation in growth rates between follicles were not reported. Therefore, to specifically evaluate follicle deviation in the absence of LH, heifers in experiment 2 were treated with a GnRHr-ant and all individual follicles were mapped throughout the expected time of follicular deviation. The hypothesis was that a deviation in growth rate between the future dominant and largest subordinate follicles, an event intimately associated with selection of a dominant follicle, requires GnRH-stimulated LH pulses.

MATERIALS AND METHODS

All animal procedures were approved by the Animal Care and Use Committee of the College of Agriculture and Life Sciences at the University of Wisconsin–Madison.

Experiment 1

Experiment 1 evaluated the effect of charcoal-stripped bFF, as a source of follicular inhibin, on FSH secretion during the preovulatory and periovulatory FSH surges. Nulliparous Holstein heifers, ranging in age from 18 to 36 mo, were observed daily for signs of estrus. Beginning at estrus, ovulation was detected by daily transrectal ultrasonography with an ultrasound scanner (Aloka SSD-900V; Aloka Co.) equipped with a 7.5 MHz transducer. Five or six days after ovulation, heifers were given two injections (12 h apart) of prostaglandin $F_{2\alpha}$ (25 mg dinoprost tromethamine; Prostamate; Phoenix Pharmaceutical). Thirty-six hours after the first prostaglandin, all heifers were administered an i.m. injection of 100 μg GnRH (Hour = 0; gonadorelin diacetate tetrahydrate; Cystorelin; Merial Limited). The 100-μg dose of GnRH was based on a previous study [9] in which the gonadotropin response to this dose of GnRH was evaluated at hourly intervals and compared to the natural GnRH/LH/FSH surge.

Heifers were randomly divided into three treatment groups (n = 6/group): 1) untreated control, 2) bFF at Hour −4, and 3) bFF at Hour 6. Treatment with bFF consisted of a single infusion of 10 ml bFF into the jugular vein, as previously utilized [13, 30]. The bFF treatment at Hour −4 was expected to reduce FSH concentration based on a previous report [30]. Treatment with bFF at Hour 6 coincided with the expected onset of the periovulatory FSH surge following a GnRH injection [9] and, based on previous studies [13], was expected to reduce FSH concentrations during the expected periovulatory FSH surge. The protocol for preparation of bFF has been described [30]. As expected, charcoal stripping in the present study reduced the concentrations of E2 from a prestripping concentration of ~400 ng/ml to a poststripping concentration of 22.8 pg/ml.

Hourly blood sampling began 6 h before GnRH treatment and continued until after the expected peak of the periovulatory FSH surge at 36 h after the GnRH injection [9]. To facilitate intensive blood sampling, each heifer was fitted with an indwelling jugular catheter as described [31]. Catheter patency was maintained by flushing with 0.9% (w/v) saline containing 100 IU/ml of sodium heparin after withdrawal of each sample. Blood samples were collected into heparinized blood tubes and refrigerated, and plasma was separated by centrifugation within 24 h of collection. The plasma supernatant was decanted into storage vials and stored at −20°C until assay.

The ovaries of each heifer were evaluated by transrectal ultrasonography every 8 h beginning 10 h before GnRH treatment (Hour 0) until detection of a ≥7.0-mm follicle. At each ultrasound examination, the location and diameter of follicles ≥3.0 mm were mapped for tracking of individual follicles. Time of ovulation was defined as the hour that the dominant follicle, present at the time of GnRH treatment, was no longer detected on the ovary.

Experiment 2

This experiment evaluated the effect of a GnRHr-ant or a GnRHr-ant plus E2 on LH and FSH secretion and development of the periovulatory follicular wave. As in experiment 1, heifers 5 or 6 days after ovulation were treated with prostaglandin, followed 36 h later by an injection of GnRH (Experimental Hour = 0). Heifers were randomly divided into three treatment groups: 1) control (n = 8), 2) GnRHr-ant (n = 8), and 3) E2 + GnRHr-ant (n = 4). The GnRHr-ant (acyline; from Dr. Robert Spirtas, NICHD, North Bethesda, MD) was administered i.m. at Hours 6, 18, and 36 and every 24 h thereafter until Hour 132. A dose of 3 μg acyline/kg body weight was used in the study. In a preliminary experiment, this dose provided inhibition of the GnRH-induced (100 μg GnRH, 1 h after acyline) LH and FSH secretion (Fig. 1). The acyline was prepared in 5% (w/v) mannitol as a 1 mg/ml solution. To evaluate the efficacy of the GnRHr-ant during the experiment, half (n = 4) of the heifers in treatments 1 and 2 were subject to two challenges with 100 μg of GnRH. The first challenge was administered at Hour 24 and the second was administered at Hour 152. In the E2 + GnRHr-ant group, injections of 0.1 mg E2-17β (i.m.) were administered every 4 h from Hours 6 to 22. This dose and frequency of E2 injections has been shown to maintain E2 near preovulatory concentrations [32]. At Hours 36, 60, 84, 108, and 132, GnRHr-ant + E2 heifers were treated with 0.1 mg of long-acting E2 cypionate (ECP; Pharmacia Corporation).

Jugular blood was sampled every hour from Hours −2 until 36 by collection procedures similar to those in experiment 1. Ultrasound examinations of the ovaries were conducted every 12 h from Hour 0 to Hour 144. Additional blood samples were acquired at the time of each ultrasound examination via the tail vein. Mapping of follicles and definitions of follicular events were similar to experiment 1, except that an additional comparison of follicular growth rates was performed between the largest follicle of the periovulatory follicular wave (F1) and the future second largest follicle (F2) to evaluate follicular deviation. The time of follicular deviation was defined as the ultrasound examination before the growth rate of F1 was significantly greater than that of F2 for two consecutive examinations.

Hormone Assays

Plasma FSH was measured by radioimmunoassay (RIA) as previously described [33, 34] using USDA-bFSH-I-2 as both the standard and radiolabeled protein and primary antibody NIDDK-anti-oFSH-I-2. The average interassay and intra-assay coefficients of variation for samples from all experiments were 8.9% and 7.0%, respectively. Mean assay sensitivity was 0.03 ng FSH/ml. Plasma LH concentrations were measured by RIA as previously described [34] using USDA-bLH-B6 as standard and radiolabeled protein and primary

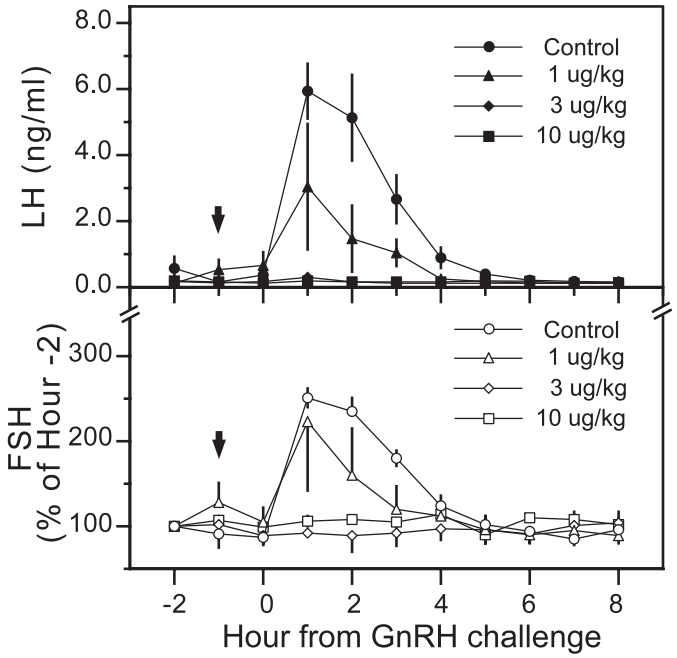

FIG 1. Mean (±SEM) circulating LH and FSH concentrations in heifers (n = 4/group) treated at Hour −1 (arrows) with various doses of the GnRHr-ant, acyline, and a GnRH challenge at Hour 0.

antibody (B225; provided by Dr. Gordon Niswender, Colorado State University). Across samples from both experiments, interassay and intra-assay coefficients of variation were 11.7% and 9.3%, respectively. Mean assay sensitivity was 0.17 ng LH/ml. Plasma E2 concentrations were measured using modifications to a commercially available E2 RIA kit (Third Generation E2 Assay Kit, Diagnostics Systems Laboratories Inc.) previously verified for use in cattle [6]. The mean assay sensitivity was 0.32 pg E2/ml.

Data Handling and Statistical Analysis

Mathematical definitions were developed for analysis of LH and FSH surges. The onset (or end) of a surge was defined as the hour before (or after) a hormone concentration had increased (or decreased) to a concentration $\geq (\leq)$ the mean plus two standard deviations of the previous two hourly samples. The EXPAND procedure in SAS (SAS, Version 7) was used to determine area under the curve during a hormone surge. The ANOVA procedure in SAS was used to compare variables among treatment groups. The MIXED procedure in SAS was used to evaluate the main effects of treatment, hour, and treatment by hour interaction on repeated measurements for plasma LH, FSH, and E2 concentrations. For all statistical analyses, a probability of $P \leq 0.05$ was considered to be significant, and probabilities between $P > 0.05$ and $P \leq 0.10$ indicated that a difference approached significance.

TABLE 1. Results (mean ± SEM) from Experiment 1 comparing the effects of bFF at Hour −4 versus combined controls and heifers treated with bFF at Hour 6 on the preovulatory LH and FSH surges in relation to GnRH treatment at Hour 0.

End point	Control	bFF at Hour −4
No. of heifers	12	6
Preovulatory LH surge		
Maximum concentration (ng/ml)	13.5 ± 0.6[a]	9.9 ± 0.9[b]
Time to peak (h)	1.7 ± 0.1	1.8 ± 0.2
Duration (h)	5.1 ± 0.2	4.8 ± 0.3
Area under curve (arbitrary units)	32.1 ± 3.2	27.1 ± 3.2
Preovulatory FSH surge		
Maximum concentration (ng/ml)	0.64 ± 0.04	0.53 ± 0.08
Time to maximum (h)	1.8 ± 0.1	1.7 ± 0.2
Duration (h)	6.0 ± 0.2	5.5 ± 0.2
Area under curve (arbitrary units)	1.4 ± 0.1	1.1 ± 0.2

[a,b] Within a row, values with different superscripts differ ($P < 0.05$).

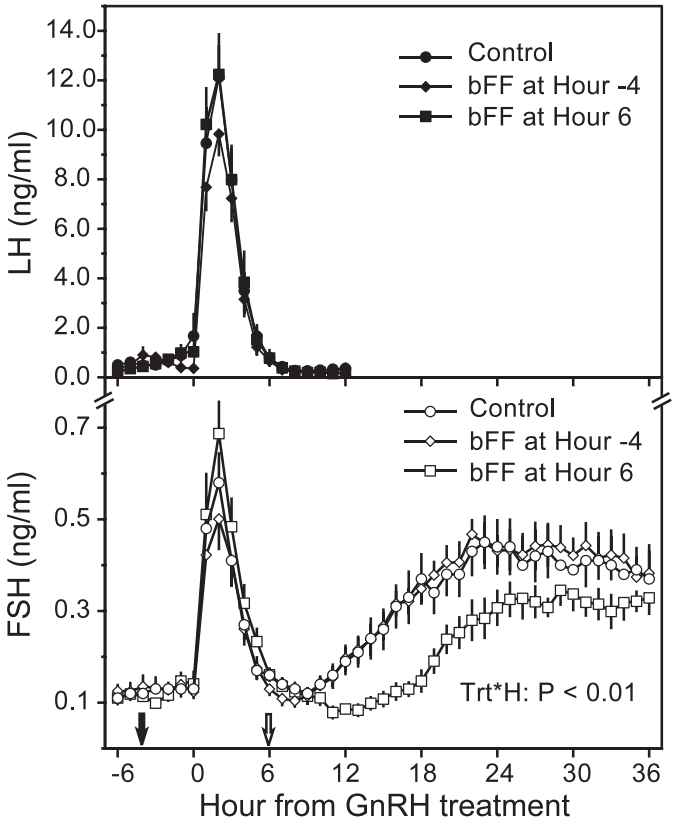

FIG 2. Mean (±SEM) circulating LH and FSH concentrations in control (n = 6) and heifers treated with bFF at Hour −4 (n = 6, solid arrow), or at Hour 6 (n = 6, open arrow), relative to GnRH treatment at Hour 0. Trt*H, treatment by hour interaction.

RESULTS

Experiment 1

Circulating FSH and LH concentrations. The beginning of FSH and LH surges was evident within 1 h after GnRH treatment in each heifer, regardless of treatment. To compare GnRH-induced LH and FSH surges from Hour −4 to Hour 6, data were combined for the six control heifers and six heifers treated with bFF at Hour 6 and compared to the six heifers treated at Hour −4 (Table 1). Relative to controls, treatment with bFF at Hour −4 did not affect ($P > 0.10$) the duration or area under the curve of the induced preovulatory LH and FSH surges (Table 1). Maximum concentration of FSH was also not altered by bFF treatment; however, the maximum concentration of LH during the preovulatory surge was reduced ($P < 0.05$) by 27%. Analysis of both LH and FSH concentrations from Hours −4 to 6 provided no indication ($P > 0.10$) of a treatment or treatment by time interaction (Fig. 2).

Treatment with bFF at Hour 6 reduced ($P < 0.05$) the minimum FSH concentration between the preovulatory and periovulatory FSH surges (0.07 ± 0.01 ng/ml, bFF at Hour 6; 0.12 ± 0.01 ng/ml, control; 0.10 ± 0.01 ng/ml, bFF at Hour −4). There was also an increase ($P < 0.01$) in the hours to onset of the periovulatory FSH surge (18.2 ± 0.06 h, bFF Hour 6; 11.0 ± 0.6 h, control; 11.7 ± 0.9 h, bFF Hour −4). Treatment with bFF at Hour 6 suppressed ($P < 0.01$) FSH concentrations from Hours 11 to 24 (Fig. 2) Concentrations of FSH in heifers treated with bFF at Hour 6 returned to concentrations similar ($P > 0.10$) to controls by Hour 25, although even after this time the FSH concentrations were

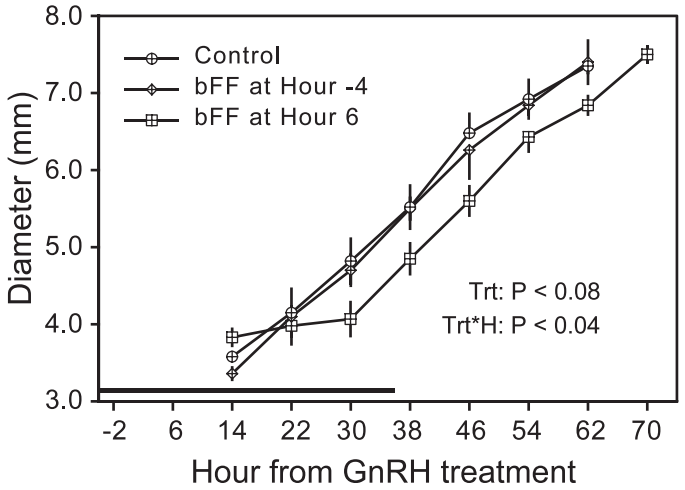

FIG 3. Growth profile of F1 in control (n = 6) and heifers treated with bFF at Hour −4 (n = 6), or at Hour 6 (n = 6), relative to GnRH treatment at Hour 0. Each point represents mean (±SEM). Solid bar denotes period of hourly blood sampling. Trt, treatment; Trt*H, treatment by hour interaction.

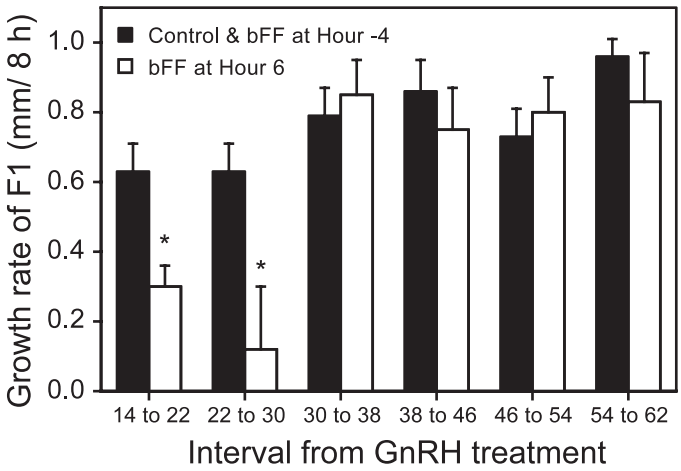

FIG 4. Mean (±SEM) growth rate of F1 during an 8-h interval in control and heifers treated with bFF at Hour −4 (combined n = 12), or bFF at Hour 6 (n = 6), relative to GnRH treatment at Hour 0. *Means are different, $P < 0.05$.

numerically and sometimes significantly lower in heifers treated with bFF at Hour 6.

Follicular development. Each heifer ovulated the dominant follicle present at the time of GnRH by Hour 30. Wave emergence (detection of follicles ≥4 mm) occurred numerically but not significantly later in heifers treated with bFF at Hour 6 (30.0 ± 4.1 h) than control (23.3 ± 3.2 h) or Hour −4 (24.7 ± 1.7 h) heifers. There were significant effects of treatment and treatment by hour interaction for the growth profile of F1 (Fig. 3). The diameter of F1 was reduced ($P < 0.05$) between Hours 30 and 62 by bFF treatment at Hour 6 (Fig. 3). Analysis of follicle growth rate indicated no difference between control and heifers treated with bFF at Hour −4, and these 12 heifers were combined for further analysis. Growth rate of F1 was less ($P < 0.05$) during Hours 14–30 in heifers treated with bFF at Hour 6 than in the other two groups and thereafter growth rate was parallel in the three groups (Figs. 3 and 4).

Experiment 2

Circulating FSH, LH, and E2 concentrations. Prior to GnRH injection, circulating concentrations of LH, FSH, and E2 were similar ($P > 0.10$) for the three treatment groups (Table 2 and Fig. 5). Each of the 20 heifers displayed a preovulatory LH and FSH surge within 1 h after GnRH injection, and LH and FSH concentrations were returning toward mean basal concentrations by Hour 6 (Fig. 6). After GnRH treatment (Hour 0) but prior to differential treatments at Hour 6, circulating E2 concentrations were elevated in heifers from all three groups (Fig. 5). Treatment with GnRHr-ant alone did not alter circulating E2 concentrations from Hours 6 to 34 after treatment. However, as expected, circulating E2 concentrations increased ($P < 0.01$) in the E2 + GnRHr-ant group beginning at Hour 10 until the final sample at Hour 34. Circulating LH concentrations were suppressed beginning 4 h after the initial GnRHr-ant treatment and, with the exception of the Hour 22 time point, remained lower ($P < 0.01$) in the GnRHr-ant and E2 + GnRHr-ant groups from Hours 10 to 24 after the GnRH-induced preovulatory LH surge (Fig. 6). Surprisingly, circulating concentrations of FSH during the periovulatory FSH surge were increased by GnRHr-ant between Hours 14 and 24, relative to control. In contrast, treatment with E2 + GnRHr-ant decreased circulating FSH, relative to control, and prevented the periovulatory increase in FSH concentrations (Fig. 6).

To confirm efficacy of the GnRHr-ant, at Hours 24 and 152, GnRH challenges were administered to four heifers in both the control and GnRHr-ant groups. The GnRH challenge at Hour 24 increased ($P < 0.01$) LH and FSH concentrations in control heifers but not GnRHr-ant-treated heifers (Fig. 7). By Hour 30, LH and FSH concentrations in GnRH-treated control heifers were similar to those in heifers not receiving the GnRH challenge, and thereafter the groups were combined for further analyses.

There was a significant effect of treatment on LH concentrations from Hours 36 to 144, with control heifers displaying consistently greater LH concentrations than either GnRHr-ant or E2 + GnRHr-ant heifers (Fig. 8). In control heifers, an increase ($P < 0.01$) in LH concentrations occurred between Hours 36 and 48. Thereafter, concentrations stabilized and then declined ($P < 0.01$) between Hours 84 and 132. In heifers treated with E2 + GnRHr-ant, LH concentrations increased ($P < 0.01$) between Hours 36 and 48 and then remained constant for the remainder of the experiment. An increase in LH concentrations was not apparent ($P > 0.10$) in heifers treated with GnRHr-ant alone.

Between Hours 36 and 144, FSH concentrations tended to be different ($P = 0.09$) among treatment groups, and there was a treatment by hour interaction ($P < 0.001$). In both control and GnRHr-ant groups, FSH declined between Hours 30 and 48. However, FSH concentrations were consistently greater in GnRHr-ant than control heifers. In the GnRHr-ant + E2 heifers, FSH was suppressed from Hours 30 to 36, but then rebounded between Hours 36 and 120 (Fig. 8). A GnRH challenge at Hour 152 also increased ($P < 0.01$) LH (Fig. 9) and FSH (data not shown) concentrations in control heifers but not in heifers treated with GnRHr-ant.

Follicular development. By Hour 30, each of the 20 heifers ovulated the dominant follicle present at the time of GnRH treatment. Time to detection of F1 at 4.0 and 5.0 mm was not influenced by GnRHr-ant treatment; however, E2 + GnRHr-ant treatment delayed ($P < 0.05$) emergence of F1 at 4.0 and 5.0 mm by ~20 and 30 h, respectively (Table 2). The F1 grew beyond 6.0 mm in only 50% of the E2 + GnRHr-ant heifers

TABLE 2. Experiment 2 results (mean ± SEM) comparing LH and FSH concentrations before GnRH treatment at Hour 0 or after beginning of GnRHr-ant and GnRHr-ant + E2 treatment at Hour 6. End points for follicle development are shown in relation to GnRH treatment (Hour 0).

End point	Control	GnRHr-ant	GnRHr-ant + E2
No. of heifers	8	8	4
LH before Hour 0 (ng/ml)	0.85 ± 0.09	0.86 ± 0.13	0.86 ± 0.05
FSH before Hour 0 (ng/ml)	0.11 ± 0.01	0.12 ± 0.01	0.10 ± 0.01
Minimum FSH after Hour 6 (ng/ml)	0.13 ± 0.02[a]	0.13 ± 0.01[a]	0.07 ± 0.01[b]
Periovulatory FSH surge			
Time to onset (h)	9.9 ± 0.5[a]	10.1 ± 0.2[a]	32.8 ± 1.7[b]
Maximum concentration (ng/ml)	0.31 ± 0.04[a]	0.37 ± 0.02[a]	0.13 ± 0.02[b]
Area under curve (arbitrary units)*	3.3 ± 0.5[a]	4.1 ± 0.3[a]	0.6 ± 0.1[b]
Percentage of heifers with F1			
≥5.0 mm (%)	100	100	100
≥6.0 mm (%)	100	100	50
≥7.0 mm (%)	100	75	50
≥8.0 mm (%)	100	50	50
≥9.0 mm (%)	100	0	0
Hours to detection of F1 at			
≥4.0 mm	27.0 ± 1.9[a]	27.0 ± 3.0[a]	48.0 ± 11.0[b]
≥5.0 mm	37.5 ± 2.7[a]	39.0 ± 1.9[a]	69.0 ± 11.4[b]
≥6.0 mm†	48 ± 2.3	54.0 ± 5.0	102 ± 4.2
≥7.0 mm†	63.0 ± 3.0	68.0 ± 5.8	126.0 ± 12.7
≥8.0 mm†	76.5 ± 3.1	84.0 ± 6.0	144.0 ± 0.0
Diameter of F1 at Hour 30 (mm)‡	5.1 ± 0.2[a]	5.0 ± 0.3[a]	4.0 ± 0.5[b]
Maximum diameter of F1 (mm)§	13.3 ± 0.5[a]	7.7 ± 0.3[b]	6.7 ± 0.8[b]

[a,b] Within a row, values with different superscripts differ ($P < 0.05$).
* Determined from onset of periovulatory surge to last hourly sample at Hour 36.
† ANOVA comparison includes only control and GnRHr-ant groups.
‡ Timing coincides with ovulation of the dominant follicle induced by exogenous GnRH (Hour 0).
§ Defined as the largest diameter attained by F1 within the 144-h period.

compared to 100% in the other groups. The growth rate of F1 (Fig. 10) did not differ ($P > 0.10$) between control and GnRHr-ant groups between Hours 24 and 36 (control: 1.11 ± 0.11 vs. GnRHr-ant: 1.34 ± 0.35 mm/12 h); Hours 36 and 48 (control: 1.20 ± 0.17 vs. GnRHr-ant: 1.04 ± 0.35 mm/12 h); and Hours 48 and 60 (control: 0.75 ± 0.1 vs. GnRHr-ant: 0.71 ± 0.1 mm/12 h). Thereafter, growth rate of F1 was different between groups (Fig. 10) between Hours 60 and 72 (control: 0.54 ± 0.17 vs. GnRHr-ant: 0.13 ± 0.2 mm/12 h; $P < 0.10$); Hours 72 and 84 (control: 1.14 ± 0.21 vs. GnRHr-ant: 0.26 ± 0.15 mm/12 h; $P < 0.01$); and Hours 84 and 96 (control: 0.86 ± 0.13 vs. GnRHr-ant: 0.39 ± 0.1 mm/12 h; $P < 0.01$). These differences

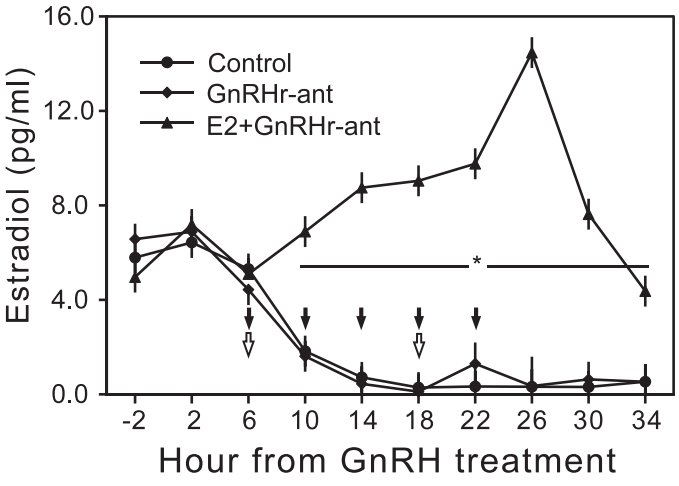

FIG 5. Mean (±SEM) circulating E2 concentrations in control heifers and heifers treated with GnRHr-ant or E2 + GnRHr-ant. Control and GnRHr-ant profiles represent the average of n = 4 randomly selected individuals. Solid arrows indicate E2 treatments and open arrows indicate GnRHr-ant treatments. *$P < 0.01$.

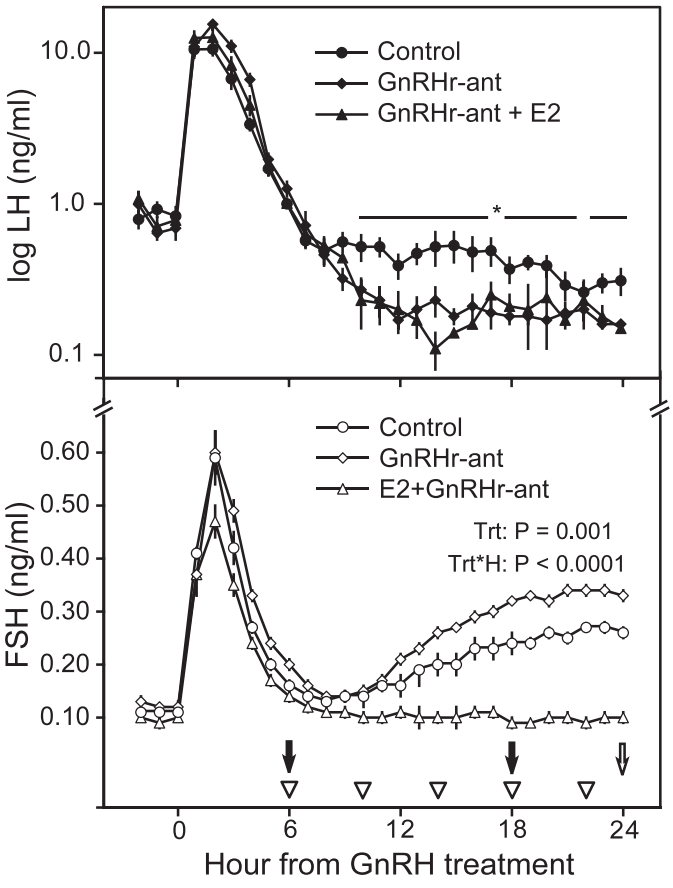

FIG 6. Mean (±SEM) circulating LH and FSH concentrations in control (n = 8) and heifers treated at Hours 6 and 18 (solid arrows) with GnRHr-ant (n = 8), or E2 (open, inverted triangles) plus GnRHr-ant at Hours 6 and 18 (n = 4). Open arrow indicates time of GnRH challenge (see Fig. 7). Trt, treatment; Trt*H, treatment by hour interaction. *$P < 0.01$ for LH.

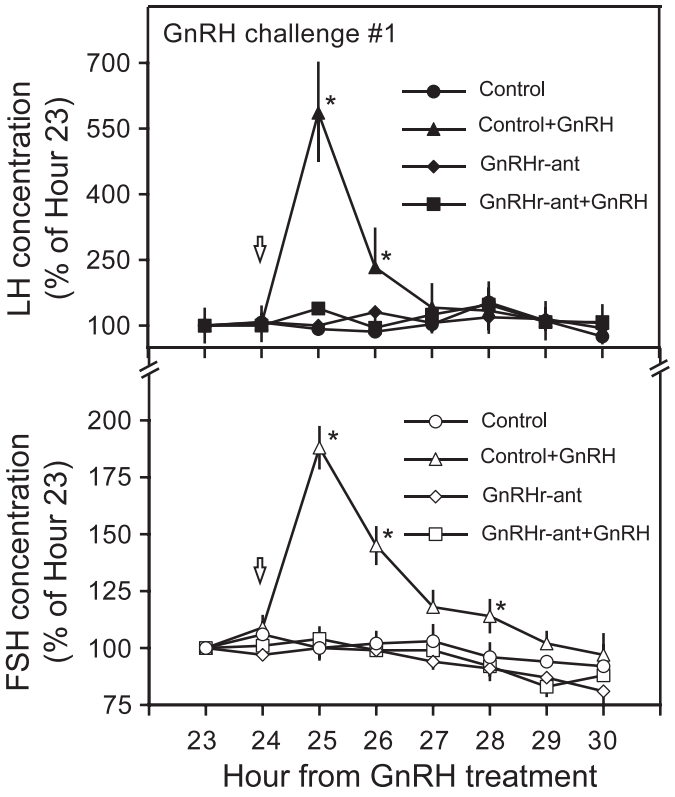

FIG 7. Mean (±SEM) circulating LH and FSH concentrations (expressed as a percentage of Hour 23) in heifers (n = 4/group) treated as control, control plus GnRH challenge, GnRHr-ant, or GnRHr-ant plus GnRH challenge. The GnRH challenge was administered at Hour 24 as indicated by the arrows. *$P < 0.05$, among lines within time.

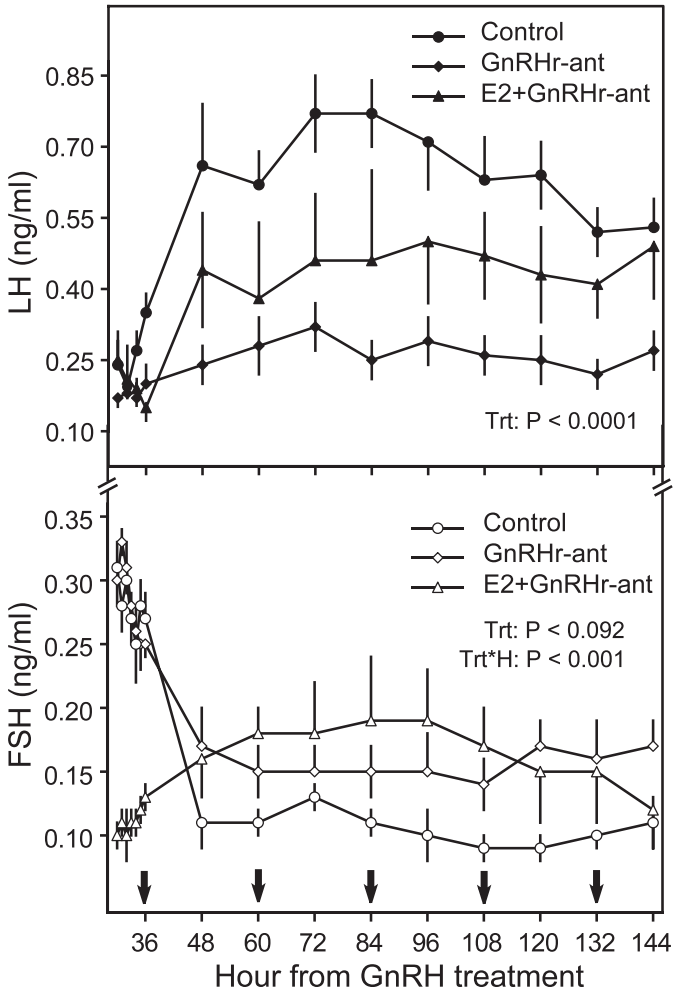

FIG 8. Mean (±SEM) circulating LH and FSH concentrations in control (n = 8) and heifers treated with GnRHr-ant (n = 8) or E2 + GnRHr-ant (n = 4). Arrows indicate times of GnRHr-ant or E2 + GnRHr-ant treatment. For clarity, LH concentrations are shown at 2-h intervals between Hours 30 and 36. Trt, treatment; Trt*H, treatment by hour interaction.

in growth rate persisted until Hour 144. In control, 100% of the heifers grew an F1 ≥9.0 mm, and 0% of heifers in the GnRHr-ant group grew an F1 ≥9.0 mm. At 48 h, no heifers in the GnRHr-ant + E2 group had grown a follicle ≥5.0 mm (largest 4.1 mm), whereas all other heifers had follicles ≥5.0 mm at this time point. At 96 h, the GnRHr-ant + E2 continued to have very small (<5.0 mm) follicles, with only 2 heifers growing follicles of 5.2 and 6.3 mm, which eventually grew to 8.0 and 8.2 mm, respectively, sizes similar to those grown by GnRHr-ant heifers.

Based on the average growth rate of F1 and F2, follicular deviation in the control group occurred at Hour 72 (Fig. 10). Between Hours 60 and 72, the growth rates of F1 and F2 were not different (F1: 0.54 ± 0.2 vs. F2: 0.6 ± 0.1 mm/12 h; $P > 0.10$), but growth rates differed at Hours 72 to 84 (F1: 1.14 ± 0.2 vs. F2: 0.23 ± 0.2 mm/12 h; $P < 0.01$) and Hours 84 to 96 (F1: 0.86 ± 0.1 vs. F2: 0.13 ± 0.2 mm/12 h; $P < 0.01$). This significant deviation in growth rate continued until the final examination at Hour 144. A comparison of F1 and F2 growth rates during a similar time interval in the GnRHr-ant group provided no indication of a deviation in growth rates between F1 and F2: Hours 60 to 72 (F1: 0.13 ± 0.2 vs. F2: 0.11 ± 0.3 mm/12 h; $P > 0.10$); Hours 72 to 84 (F1: 0.26 ± 0.2 vs. F2: 0.14 ± 0.2 mm/12 h; $P > 0.10$); Hours 84 to 96: (F1: 0.39 ± 0.1 vs. F2: −0.06 ± 0.2 mm/12 h; $P < 0.01$); and Hours 96 to 108 (F1: −0.24 ± 0.2 vs. F2: −0.19 ± 0.2 mm/12 h; $P > 0.10$).

Figure 11 shows the diameter of the corpus luteum and the circulating progesterone concentrations in the three treatment groups. The corpus luteum grew at the same rate following ovulation with a similar increase in circulating progesterone

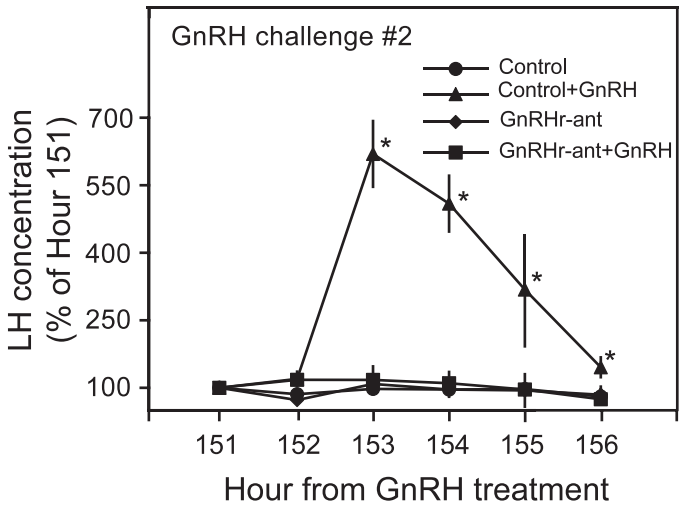

FIG 9. Mean (±SEM) circulating LH concentrations (expressed as a percentage of Hour 151) in heifers (n = 4/group) treated as control, control plus GnRH challenge, GnRHr-ant, or GnRHr-ant plus GnRH challenge. GnRH challenge #2 was administered at Hour 152, 20 hours after the last GnRHr-ant treatment at Hour 132. *$P < 0.05$, among lines within time.

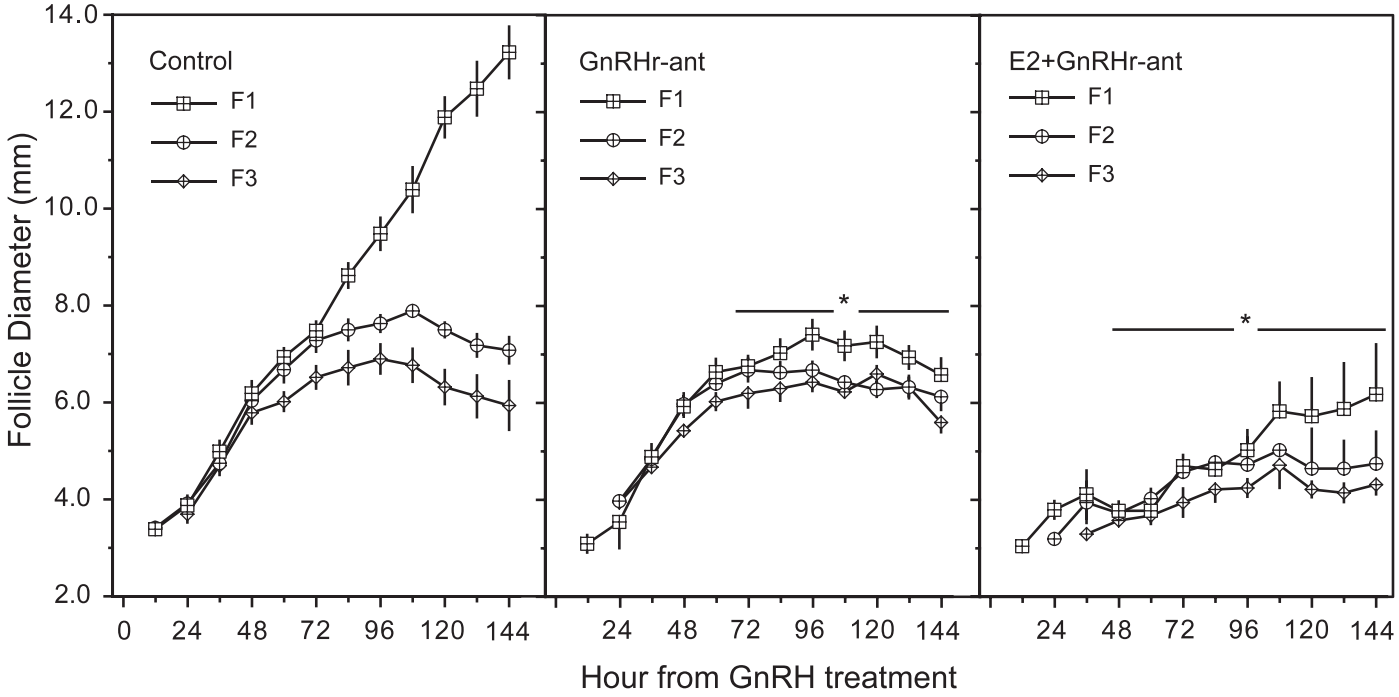

FIG 10. Growth profile of the largest (F1), second largest (F2), and third largest follicle (F3) in control (n = 8) and heifers treated with GnRHr-ant (n = 8) or estradiol plus GnRHr-ant (E2+GnRHr-ant, n = 4). Each point represents the mean (±SEM). *Time points where F1 diameter is significantly smaller than control ($P < 0.05$).

concentrations. Following treatment with prostaglandin F2α at Hour 144, there was a similar timing of luteal regression and decreasing progesterone concentrations.

DISCUSSION

These studies provide insights into hormonal regulation of the preovulatory and periovulatory FSH surges and on follicular development during this period. It was clear that there are distinctive and acute roles for GnRH, inhibin, and E2 in regulation of the 2 FSH surges in heifers. The GnRH receptor antagonist, acyline, effectively blocked GnRH-stimulated LH and FSH secretion during the preovulatory gonadotropin surge but did not suppress FSH concentrations during the periovulatory FSH surge. This result allowed us to consider the role of LH in development of the follicular wave and selection of a dominant follicle. The following conclusions seem reasonable: 1) the GnRH-induced preovulatory FSH surge was not regulated by inhibin, and 2) the periovulatory FSH surge was caused by the drop in follicular inhibin and E2 that followed the LH surge and was independent of GnRH action. The periovulatory FSH surge was essential for development of the first follicular wave. Finally, these results further support and expand our understanding of the changes in gonadotropin requirements near the time of follicular deviation in cattle.

In experiment 1, treatment with bFF before GnRH injection did not block or suppress the GnRH-dependent, preovulatory FSH surge. These results are consistent with the concept that GnRH-dependent FSH secretion is not acutely regulated by follicular inhibin, indicating that the preovulatory FSH surge is induced despite strong FSH inhibitory signals of ovarian origin. In a study using intact anestrous ewes, maximum concentrations of FSH following a GnRH injection were not significantly reduced after 4 days of bFF treatment [35]. However, because endogenous inhibin was not removed during

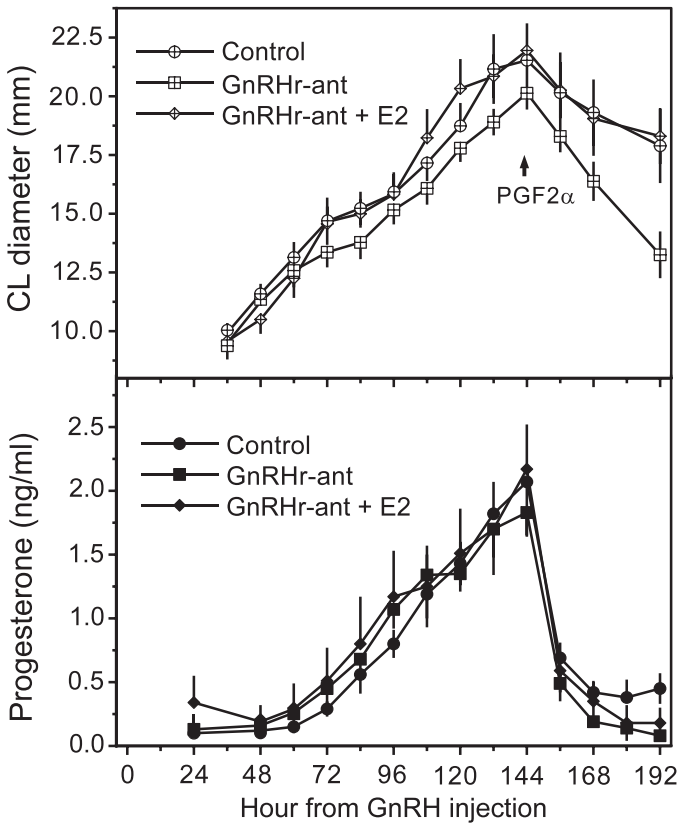

FIG 11. Diameter of the corpus luteum (CL) and circulating progesterone concentrations in heifers in the three treatment groups starting at 24 h after GnRH treatment. All heifers were treated with prostaglandin F2α (PGF2α) at Hour 144 and evaluation continued until Hour 192.

our study, there remains the possibility that endogenous inhibin was already having maximal inhibitory effects on FSH, producing a refractoriness to exogenous inhibin. In contrast to the lack of effect on FSH, bFF treatment at Hour −4 caused a slight but significant reduction in the maximum concentrations of LH during the GnRH-induced preovulatory surge. This was unexpected because a previous study reported no effect of inhibin on synthesis of LH β subunit mRNA even though FSH β subunit was suppressed within 6 h of inhibin treatment [36].

Treatment with GnRHr-ant following the preovulatory LH/FSH surges did not decrease the periovulatory circulating FSH concentrations. The dose of GnRHr-ant used in this study inhibited LH secretion even in response to a very high dose of exogenous GnRH (100 μg). These data indicate that the periovulatory FSH surge in heifers occurs independently of acute GnRH-receptor stimulation at the pituitary. These results further support the physiological model [7, 8, 37] that the periovulatory FSH surge is due to a decline in ovarian inhibitory factors (inhibin and E2) after the preovulatory LH surge and not to changes in GnRH stimulation. This model is consistent with the temporal relationships between FSH, E2, and inhibin during the periovulatory period [10, 38]. During the bovine preovulatory period, inhibin A and E2 increase with a corresponding decline in circulating FSH prior to the abrupt increase in FSH and LH during the preovulatory gonadotropin surge [10]. The preovulatory gonadotropin surge inhibits follicular expression of inhibin A (inha) and aromatase (Cyp-19A1), leading to an abrupt decrease in circulating inhibin A and E2 initiating the rise in FSH of the periovulatory surge [10]. Using estrus as a reference point, a previous study indicated that the periovulatory reduction in inhibin is important for the periovulatory FSH surge [13]. The present results further indicate that preovulatory concentrations of E2 can also potently and directly inhibit FSH secretion from the pituitary, suggesting that the E2 decline during this period is equally important for permitting the periovulatory FSH surge. It has been previously shown that E2 can have direct inhibitory effects on FSH at the level of the pituitary [39, 40]. The GnRHr-ant model provided a unique in vivo system to evaluate the direct role of E2 on FSH because the GnRHr-ant negates the confounding effect of positive E2 feedback on GnRH secretion.

Based on cell culture studies, LH is primarily targeted to the regulated secretory pathway in pituitary cells [41], whereas FSH is primarily targeted to what has been termed the constitutive secretory pathway [37, 42]. This targeting is not due to the sulfated *N*-linked oligosaccharides that are characteristic of LH and not FSH [43] but appears to be due to the seven amino acids at the carboxyl-terminal tail of LH that form a hydrophobic sorting signal directing LH to regulated secretory granules [41, 43]. Removal of this heptapeptide from LH increased constitutive expression of the altered LH [41], whereas adding this heptapeptide to FSH reroutes FSH to the regulated secretory pathway [43]. Nevertheless, our results indicate that sufficient FSH enters the regulated secretory pathway to produce the preovulatory FSH surge even though it is also clear that substantial FSH is secreted constitutively during the periovulatory FSH surge. Peak FSH concentrations during the natural preovulatory FSH surge are similar to peak FSH concentrations during the periovulatory FSH surge [9]. However, the preovulatory FSH surge is greater if induced by treatment with 100 μg GnRH than during the natural preovulatory FSH surge [9]. Whether the FSH secreted in these two surges is derived from different cells or from different compartments or secretory mechanisms in the same cells has not yet been defined. The finding that treatment with GnRHr-ant increased rather than decreased FSH concentrations during the periovulatory surge was unexpected. This result may reflect greater production or secretion of FSH by pituitary cells, perhaps due to reduced follicular androgen, E2, or inhibin in animals with suppressed LH pulses. Alternatively, targeting of pituitary FSH to the constitutive secretion pathway could be favored over the regulated pathway in GnRHr-ant treated heifers. Our study does not allow delineation of which mechanism(s) underlie the increase in FSH during the periovulatory FSH surge in heifers treated with GnRHr-ant.

In agreement with previous reports, treatment with follicular fluid suppressed circulating FSH concentrations during the periovulatory FSH surge [13, 18]. Turzillo and Fortune [13] indicated that suppression of the periovulatory FSH surge by treatment with multiple injections of bFF at 12, 24, and 36 h after the onset of estrus delayed the emergence of follicles ≥5.0 mm in the new follicular wave. In experiment 1, bFF treatment at Hour 6 did not delay emergence of the new follicular wave; however, the growth rate of F1 was reduced from Hours 14 to 30. The disparity between experiments is likely due to the single versus multiple bFF treatments. The mechanism underlying inhibition of FSH synthesis and secretion by inhibin appears to be competitive antagonism of activin receptor activation in the pituitary [8, 44, 45]. In experiment 2, treatment with E2 combined with GnRHr-ant delayed emergence of ≥5.0 mm follicles about 30 h associated with an extended (~22 h) suppression of FSH concentrations during the expected periovulatory FSH surge. Mechanistically, inhibition by E2 of FSH synthesis and secretion appears to be modulated by inhibition of pituitary synthesis of activin [46]. Thus, although FSH was suppressed in different ways, each experiment is consistent with the idea that emergence and growth rate of the periovulatory follicular wave is dependent upon increased FSH concentrations during the periovulatory surge [15].

Treatment with GnRHr-ant did not alter the timing of emergence of the follicular wave or growth rate of F1 until ~Hour 60. This implies that pituitary stimulation by GnRH is not essential to support follicle growth until follicles reach diameters between 6.6 and 8.8 mm (the range of maximum F1 diameters in the GnRHr-ant group). Relative to the control group, a significant reduction in growth rate of F1 was apparent in the GnRHr-ant group only after Hour 60, corresponding to the time when F1 reached an average diameter of 7.1 ± 0.2 mm in control and 6.8 ± 0.4 mm in the GnRHr-ant group. Furthermore, follicular deviation did not occur in the GnRHr-ant group and F1 failed to distinguish itself as a dominant follicle. The failure in the selection mechanism that resulted from the truncated growth of F1 was apparently not related to a reduction in concentrations of FSH, which remained elevated in the GnRHr-ant group, probably because of the absence of a dominant follicle.

It is likely that the inhibition of LH pulses and reduction in circulating LH concentrations in heifers treated with GnRHr-ant was responsible for precluding deviation of the dominant follicle. This result is consistent with the extensively discussed model [15, 23, 25, 27, 29, 47–49] relating deviation of a single dominant follicle and continued growth of this dominant follicle to the actions of LH. Indeed, granulosa cells of the future dominant follicle have increased expression of LH receptors near the time of selection of a dominant follicle [26–28, 47]. The increase in granulosa cell LH receptors in F1 was associated with a reduction in insulin-like growth factor-binding proteins and an increase in free insulin-like growth factor-1 and E2 [26, 49].

A secondary hypothesis in this study was that treatment with GnRHr-ant would reduce the size of and progesterone production by the corpus luteum. Surprisingly, there was no effect of GnRHr-ant on either diameter of the corpus luteum or circulating progesterone. This result is similar to a study in ewes in which treatment with a GnRHr-ant did not alter circulating progesterone concentrations [50]. However, this contrasts with a previous study in heifers in which treatment with GnRHr-ant from Days 2 to 7 of the estrous cycle decreased circulating progesterone to about 50% of the normal concentrations, although the corpus luteum developed and regressed at the normal time [51]. We also hypothesized that the corpus luteum would not regress normally in response to treatment with prostaglandin F2α, because of either lack of exposure of the corpus luteum to LH pulses or follicular E2. This hypothesis could be rejected, based on our experimental results, because prostaglandin F2α produced a similar magnitude and timing of luteal regression in all three treatment groups.

In summary, treatment with a GnRH receptor antagonist (GnRHr-ant) increased FSH concentrations during the periovulatory FSH surge, indicating that the periovulatory FSH surge resulted from an upregulation in GnRH-independent FSH secretion. Treatment with either E2 or inhibin suppressed FSH concentrations during the periovulatory surge, suggesting that the decline in follicular production of E2 and inhibin after the LH surge are important for development of the periovulatory FSH surge. Treatment with bFF did not reduce or block the preovulatory FSH surge induced by an exogenous injection of GnRH, indicating that GnRH-dependent FSH secretion is apparently not acutely regulated by follicular inhibin. Development of follicles to $\geq$5 mm during the first follicular wave was impaired by suppression of the periovulatory FSH surge using treatment with either E2 or inhibin. In contrast, GnRHr-ant had no effect on follicle development from 5 to 8 mm, indicating that the acute effects of hypothalamic GnRH secretion and LH pulses play a limited role in these stages of follicle development. Treatment with the GnRHr-ant reduced circulating concentrations of LH and prevented follicular deviation and subsequent growth of a single dominant follicle. Thus, GnRH-dependent LH secretion is a necessary component of the process that results in selection of a single dominant follicle in cattle.

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