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Immunoneutralization of Inhibin and Estradiol during the Follicular Phase of the Estrous Cycle in Cows[1]

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ABSTRACT

To investigate the physiological importance of inhibin and estradiol in the regulation of FSH secretion during the follicular phase of the estrous cycle in cows, animals were passively immunized against the two hormones. Sixteen cows were divided into four equal groups and given injections of prostaglandin $F_{2\alpha}$ (PG) i.m. twice at 8-h intervals on Day 10 of the estrous cycle (Day 0 = day of estrus) to induce luteal regression. At 48 h after the first PG injection, each group of four cows received injections of one of the following: 100 ml castrated goat serum (control serum), 100 ml antiserum against inhibin, 100 ml antiserum against estradiol, or a combination of 100 ml inhibin antiserum and 100 ml estradiol antiserum.

The LH surge occurred within 2 days after injection of the control serum or inhibin antiserum, whereas it was not detected in either of the groups passively immunized against estradiol, indicating that passive immunization against estradiol blocks a positive feedback effect of estradiol on LH secretion. There was no clear difference in basal concentrations of LH among the four groups. Injection of the inhibin antiserum resulted in a marked increase ($p < 0.01$) in plasma concentrations of FSH compared with values in the control group, while there were no significant changes in concentrations of plasma FSH after injection of the estradiol antiserum. Combined administration of the inhibin and estradiol antisera also produced a marked increase ($p < 0.01$) in plasma concentrations of FSH. The FSH response to the combined immunization was longer in duration than the FSH response to immunization with inhibin alone. Treatment with the inhibin antiserum, either alone or in combination with the estradiol antiserum, induced the growth of a large number of small (≥ 4 < 7 mm in diameter), medium (≥ 7 < 10 mm), and large (≥ 10 mm) follicles compared with numbers in the controls. No increase in the number of follicles was noted in the estradiol-immunized animals. The present results provide strong evidence that inhibin is an important factor in the inhibitory regulation of FSH secretion during the follicular phase in cows, suggesting also that estradiol has a synergistic effect with inhibin on FSH secretion.

INTRODUCTION

Estradiol is thought to be a potent ovarian factor that inhibits FSH secretion in cows. A negative correlation between plasma concentrations of estradiol and FSH is noted during the follicular phase [1–4]. Injection of estradiol at a supraphysiological dosage was found to decrease concentrations of plasma FSH in ovariectomized heifers [1, 5], and a combination of estradiol and progesterone at concentrations of the luteal phase can reduce FSH concentrations in ovariectomized heifers to levels within those observed during the estrous cycle [6].

On the other hand, it is also known that steroid-free bovine follicular fluid [2, 7–9] or highly purified bovine inhibin [9] has the ability to suppress FSH secretion in ovariectomized and cyclic heifers. A severe suppression of plasma concentrations of FSH occurred in cattle induced to superovulate with eCG [10, 11] concomitantly with dramatic increases in both bioactive and immunoreactive inhibin in the circulation [11]. These previous studies suggest the possibility that inhibin is involved in the regulation of FSH secretion in the intact cow. Recently, active immunization of heifers against synthetic peptides of the bovine inhibin α subunit [12–17] and against ovine recombinant inhibin α subunit [18] has been found to increase the number of ovulations. Among those studies [12–15, 18] in which the plasma FSH concentration was measured, several [12, 13, 18] showed a significant rise in plasma FSH after inhibin immunization, while in the others [14, 15] an apparent FSH increase could not be detected. Passive immunization experiments are needed to provide clear evidence that the decrease in endogenous inhibin directly causes the increase in FSH secretion. Our previous report demonstrated that passive immunoneutralization against inhibin during the midluteal phase of the bovine estrous cycle produced a marked increase in plasma concentrations of FSH, indicating that inhibin has an important role in the regulation of FSH secretion when estradiol secretion is low [19]. However, an endocrine role of inhibin during the follicular phase when estradiol secretion is at its highest remains an open question.

In this study, we have investigated the importance of in-

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hibin and estradiol in the regulation of FSH secretion during the follicular phase of the bovine estrous cycle by immunoneutralization of circulating inhibin and estradiol with use of anti-inhibin serum and anti-estradiol serum raised in goats.

MATERIALS AND METHODS

Inhibin Antiserum

The inhibin antiserum used in this study was raised in a castrated Saanen goat against purified bovine 32-kDa inhibin as reported previously [19]. The final titer of the batch (GB-2) of this antiserum, assessed at 50% binding of ^{125}I-labeled bovine 32-kDa inhibin (325 Ci/mmol), was 1:96 000. The antiserum recognizes the α subunit of bovine 32-kDa inhibin as shown by immunoblotting. It was previously demonstrated that administration of the inhibin antiserum at a dosage of 100 ml caused a significant rise in the plasma FSH concentration in cows weighing approximately 600 kg [19].

Estradiol Antiserum

1,3,5(10)-Estratrien-3,17β-diol-6-one 6-CMO conjugated to BSA (Steraloids, Inc., Wilton, NH), dissolved in 0.9 % (w/v) NaCl (1 mg/ml), was emulsified in an equal volume of Freund's complete adjuvant (Iatron Co. Ltd., Tokyo, Japan) and used for the primary immunization in castrated goats (2 mg conjugated estradiol/goat) by s.c. injection at multiple sites. A booster injection of 1 mg conjugate emulsified with Freund's incomplete adjuvant (Wako Pure Chemical Industries Ltd., Osaka, Japan) was administered every 4 wk, and antiserum was obtained 1 wk after each booster injection. The final titer of the batch (E11) of antiserum used in this study, assessed at 50% binding of ^{125}I-labeled estradiol (2000 Ci/mmol), was 1:4 800 000. The labeled estradiol (IM 135) was purchased from Amersham Life Science (Tokyo, Japan). Cross-reactivities, determined in vitro [20] at a final antiserum dilution of 1:4 000 000, were 100% for estradiol, 42% for estrone, 1.4% for estriol, and less than 0.1% for progesterone and testosterone.

To estimate a volume to neutralize circulating estradiol in the cows used in this study, a pilot study was conducted in two mature cows weighing approximately 500 kg each. At 48 h after the first injection of prostaglandin $F_{2\alpha}$ (PG; 15 mg, twice) on Day 10 of the estrous cycle (Day 0 = estrus), animals were given an i.v. bolus injection of 50 ml estradiol antiserum. After the injection of estradiol antiserum, plasma samples were collected every 2 h for 48 h; the sampling rate was then reduced to every 8 h for 48 h. Ovarian response was determined by an ultrasound scanner. Injection of 50 ml estradiol antiserum blocked the LH surge and ovulation in these animals. The results suggested that administration of 50 ml estradiol antiserum blocked a positive feedback effect of estradiol on LH secretion, and a dosage of 100 ml was used in the present study to ensure the immunoneutralization of circulating estradiol.

Experimental Design

Ten Japanese brown and six Japanese black cows (3–7 yr old), clinically normal with regular estrous cycles, were used. The animals were divided into four groups (n = 4 each) with similar mean group weights: control (523 ± 34 kg [mean ± SEM]), estradiol-immunized (531 ± 12 kg), inhibin-immunized (526 ± 12 kg), and combined-immunized (520 ± 29 kg) groups. Cannulae were inserted into their jugular veins 1 day before each experiment under tranquilization with 0.04 mg xylazine (Celactal; Bayer Japan Co., Tokyo, Japan). On Day 10 of the estrous cycle, 15 mg of PG was administered i.m. twice at 8-h intervals. The time of the first PG injection was defined as 0 h. At 48 h, the animals of each group received an i.v. bolus injection of 100 ml castrated goat serum (control group), 100 ml inhibin antiserum (inhibin-immunized group), 100 ml estradiol antiserum (estradiol-immunized group), or a combination of 100 ml inhibin antiserum and 100 ml estradiol antiserum (combined-immunized group) through an indwelling jugular cannula. Estrous behavior (standing estrus) was checked every 4 h between 48 and 96 h and then every 8 h until 144 h. Blood samples were collected via jugular cannulae every 8 h between −24 h and 48 h. After the injection of control serum or antisera at 48 h, blood samples were taken every 2 h for 16 h (64 h); then the sampling interval was prolonged to every 4 h until 96 h, to every 8 h until 144 h, and finally to every 24 h to the end of the experiment (216 h). Plasma was removed after centrifugation and stored at −30°C until required for assays for FSH, LH, estradiol, and progesterone. The inhibin- and estradiol-binding activity and the concentrations of unbound estradiol antiserum in plasma following passive immunization were also determined.

Determination of Ovarian Response

The population of ovarian follicles in each group was examined at 24-h intervals, from 24 h before the first PG injection to 9 days after the first PG injection, with use of an ultrasound scanner (Echo Camera 210 DX II, Tokyo, Japan) as reported previously [3]. Since growth and regression of individual follicles could not be identified because of the appearance of a large number of growing follicles in the inhibin- and combined-immunized groups (see *Results*), data on follicular growth in each treatment were presented by showing the changes in number of the three categories of follicles classified according to their mean diameter (small, ≥ 4 < 7; medium, ≥ 7 < 10; large, ≥ 10 mm).

RIA of FSH, LH, Estradiol, and Progesterone

Plasma concentrations of FSH were measured by RIA [21] using anti-bovine FSH β-subunit antiserum (USDA-5-pool),

USDA-FSH-BP3 for radioiodination, and USDA-FSH-B1 as a reference standard. Plasma concentrations of LH were measured by RIA [22] using anti-ovine LH serum (USDA-309–684P), USDA-bLH-I-1 for radioiodination, and USDA-bLH-B-1 as a reference standard (RIA materials for bovine FSH and LH were supplied by Dr. D.J. Bolt, USDA, Beltsville, MD). The sensitivities of the assays for LH and FSH, based on a 95% confidence limit of the zero standard, were 0.006 ng/tube (0.06 ng/ml) and 0.24 ng/tube (1.2 ng/ml), respectively. The intra- and interassay coefficients of variation, calculated according to the methods of Rodbard [23], were 6.2% and 12.9% for LH and 4.8% and 8.7% for FSH, respectively.

Plasma concentrations of estradiol and progesterone were determined as described previously [24] through the use of antisera to estradiol-17β (GDN 244; [25], supplied by Dr. G.D. Niswender, Department of Physiology and Biophysics, Colorado State University, Fort Collins, CO) and progesterone (GDN 337; [26], supplied by Dr. G.D. Niswender). ^{125}I-Labeled estradiol (IM 135) and progesterone (IM 140) were purchased from Amersham Life Science (Tokyo, Japan). For the assay of estradiol, 1-ml plasma samples, placed in 13 × 100-mm glass tubes, were extracted with 3 ml anhydrous diethyl ether. The ether phase was decanted into 12 × 75-mm tubes and evaporated. To remove substances that interfere with the estradiol assay, a solution of a mixture of 2 ml n-hexane and 0.5 ml 50% methanol (v/v) was added, and the tubes were mixed. After aspiration of the hexane phase, the methanol phase was dried with nitrogen gas and redissolved in 0.1 ml PBS (50 mmol/L, pH 7.5) containing 1% (w/v) BSA (BSA-PBS), and the solution was used for the estradiol RIA. For the assay of progesterone, 0.025–0.1-ml plasma samples, pipetted into 13 × 100-mm tubes that were then filled up to 0.5 ml with distilled water, were extracted with 2.5 ml anhydrous diethyl ether. After the ether phase was decanted into 12 × 75-mm tubes and evaporated, the dried residue was redissolved in 0.1 ml BSA-PBS, and the solution was used for progesterone RIA. The sensitivities of the assays for estradiol and progesterone were 0.32 pg/tube (0.32 pg/ml) and 2.5 pg/tube (25 pg/ml), respectively. The intra- and interassay coefficients of variation were 5.1% and 9.1% for estradiol and 4.5% and 12.1% for progesterone, respectively.

Determination of the Ability to Bind Inhibin and Estradiol in Plasma Following Bolus Injection of the Antisera

Changes in inhibin-binding activity in plasma of the inhibin-immunized animals were determined by measuring the binding of ^{125}I-labeled inhibin (325 Ci/mmol) as reported previously [19]. Changes in estradiol-binding activity in plasma were determined in the following manner. Plasma obtained at various times after injection of the estradiol antiserum was diluted 1:30 with BSA-PBS. Plasma dilution (100 μl) and BSA-PBS (100 μl) were incubated for 24 h at 4°C with ^{125}I-labeled estradiol (5000 cpm/100 μl; 2000 Ci/mmol) in 10 × 75-mm glass tubes (total volume 300 μl). Bound tracer was then separated by adding 100 μl PBS that contained 1% (w/v) bovine gamma globulin and 500 μl PBS containing 25% (w/v) polyethylene glycol. After centrifugation at 1700 × *g* for 30 min, radioactivity in the precipitate was counted. Estradiol-binding activity was expressed as a percentage of the total counts added.

Determination of Changes in Plasma Concentrations of Unbound Estradiol Antiserum Following Injection of the Antiserum

Since the estradiol-binding activity showed no apparent fluctuation during the 7 days after injection of the estradiol antiserum (see *Results*), concentrations of circulating unbound estradiol antiserum were further determined as described by Morishige et al. [27] in order to examine accurately the change in the capacity to immunoneutralize circulating estradiol. Estradiol antiserum, serially diluted with BSA-PBS, was used as a reference standard (7.8×10^{-5} to 0.01 μl/ml). Plasma samples obtained at various times after injection of the estradiol antiserum were diluted 1:40 000 with BSA-PBS. One hundred microliters of standards or plasma dilution was incubated for 24 h at 4°C with ^{125}I-labeled estradiol (5000 cpm/100 μl; 2000 Ci/mmol) in 10 × 75-mm glass tubes (total volume 200 μl). Bound tracer was then separated by adding 100 μl PBS that contained 1% (w/v) bovine gamma globulin and 500 μl PBS containing 25% (w/v) polyethylene glycol. After centrifugation at 1700 × *g* for 30 min, radioactivity in the precipitate was counted. A dose-response curve of the standard was obtained between 7.8×10^{-5} and 6.25×10^{-4} μl/ml (binding; 34% to 70%). Plasma samples, diluted at 1:40 000, showed 26–50% of the binding with labeled estradiol. The results are expressed as μl equivalents of estradiol antiserum/ml plasma.

Statistics

Results were subjected to analysis of variance for repeated measures [28]. When a significant effect was obtained with analysis of variance, the significance of the difference between two means was tested by Student's *t*-test. When more than two means were compared, the significance of the difference between means was determined by Duncan's Multiple Range test. All data were analyzed through use of the General Linear Model Procedure of the Statistical Analysis Systems [29]. A value of $p < 0.05$ was considered to be significant.

RESULTS

Estrous Behavior

In the control group, two animals showed estrous behavior (standing estrus) between 88 and 112 h after the first

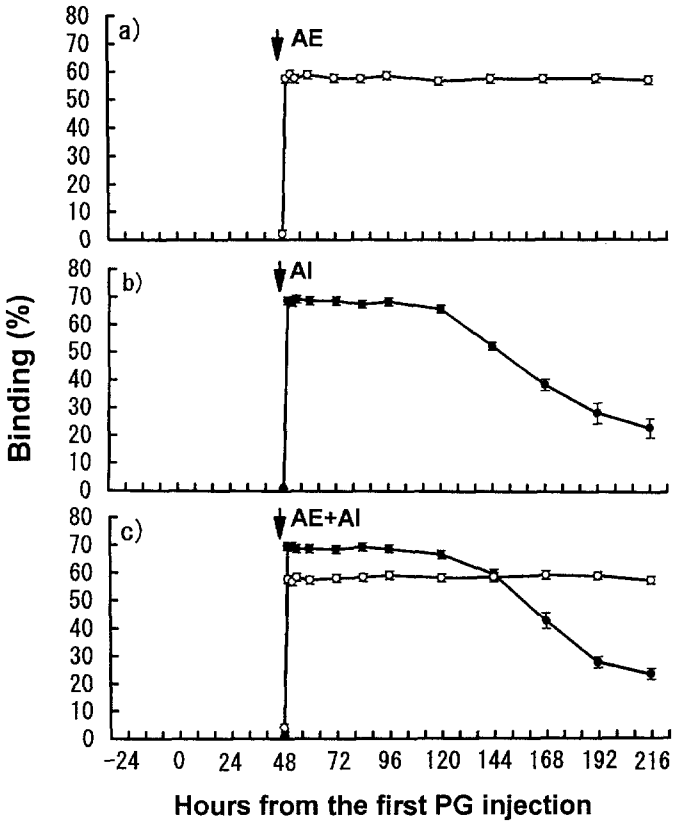

FIG. 1. Estradiol-binding activity (open circles), at a final dilution of 1:90, or inhibin-binding activity (solid circles), at a final dilution of 1:45, in plasma of cows that received an i.v. bolus injection of (a) 100 ml estradiol antiserum (AE), (b) 100 ml inhibin antiserum (AI), or (c) 100 ml estradiol antiserum and 100 ml inhibin antiserum (AE + AI) at 48 h after the first PG injection (Time 0) on Day 10 of the estrous cycle (Day 0 = day of estrus). Arrows indicate injections of antisera. Values are means ± SEM for four cows.

PG injection, another between 84 and 112 h, and the fourth between 76 and 92 h. The duration of estrous behavior in each animal in the inhibin-immunized group was 76 to 108 h, 88 to 116 h, 92 to 114 h, and 96 to 120 h. All animals in the estradiol- and combined-immunized groups, on the other hand, showed no estrous behavior during the period of observation (48–144 h).

Hormone-Binding Activities in the Circulation Following Passive Immunization

The estradiol-binding activity in plasma of the estradiol- and combined-immunized animals, at a final plasma dilution of 1:90, was about 60% at 2 h after injection of the antibody and then showed no change during the period of the experiment (Fig. 1, a and c). The inhibin-binding activity in plasma of inhibin-immunized animals, at a final plasma dilution of 1:45, was about 70% by 2 h, and this level was sustained for 72 h (120 h following the first PG injection) (Fig. 1b). The binding activity significantly ($p < 0.01$) decreased to 52.6 ± 0.9% (mean ± SEM) at 144 h and further declined at 168 h. Inhibin-binding activity in the combined-immunized animals showed a pattern quite similar to that in the animals passively immunized against inhibin alone (Fig. 1c).

Changes in Plasma Concentrations of Unbound Estradiol Antiserum Following Injection of the Antiserum

At 2 h after passive immunization, concentration of unbound estradiol antiserum in plasma was 5.6 ± 0.3 (mean ± SEM) μl/ml in the estradiol-immunized group (data not shown). Unbound antiserum levels in the estradiol-immunized group significantly ($p < 0.01$) decreased to 4.1 ± 0.2 μl/ml 24 h later (72 h) and further declined ($p < 0.01$) at 96 h. At the end of the experiment (216 h), the concentration of unbound estradiol antiserum was 2.2 ± 0.1 μl/ml. Changes in concentrations of unbound estradiol antiserum in the combined-immunized group showed a pattern quite similar to that in the animals passively immunized against estradiol alone.

Plasma Concentrations of LH

Concentrations of plasma LH in all groups were under 2 ng/ml except at the time of the preovulatory LH rise, and there were no significant differences in the basal concentrations of plasma LH among the four groups (Fig. 2). A significant ($p < 0.01$) increase in plasma LH was noted at 88.0 ± 2.8 h (mean ± SEM) after the first PG injection in the control group and at 89.0 ± 3.0 h in the inhibin-immunized group. The peak value of the LH rise in the controls was 8.6 ± 0.7 ng/ml, which was not significantly different from the value in the inhibin-immunized animals (9.1 ± 1.6 ng/ml) (Fig. 3a). No animal in the two groups that had received an injection of the estradiol antiserum showed any successive increases in plasma LH greater than 3 ng/ml.

Plasma Concentrations of FSH

Following the injection of castrated goat serum at 48 h, plasma concentrations of FSH did not significantly change during the period before the onset of the LH rise (48–68 h) (Fig. 4a). In the estradiol-immunized animals, there were no significant changes in plasma concentrations of FSH throughout the study (Fig. 4b). In contrast, treatment with the inhibin antiserum or the combination of inhibin and estradiol antisera resulted in a marked increase ($p < 0.01$) in plasma concentrations of FSH during the same period (48–68 h) compared with values for the control group (Fig. 4, c and d). In both the inhibin- and combined-immunized animals, concentrations of FSH significantly increased ($p < 0.01$) at 12 h after injection of the antibody compared to the levels just before treatment. A significant ($p < 0.05$) rise in plasma FSH, coincidentally with the LH increase, was noted on the day of estrus in the control and inhibin-immunized animals (Fig. 3b). The peak value of the FSH rise in inhibin-

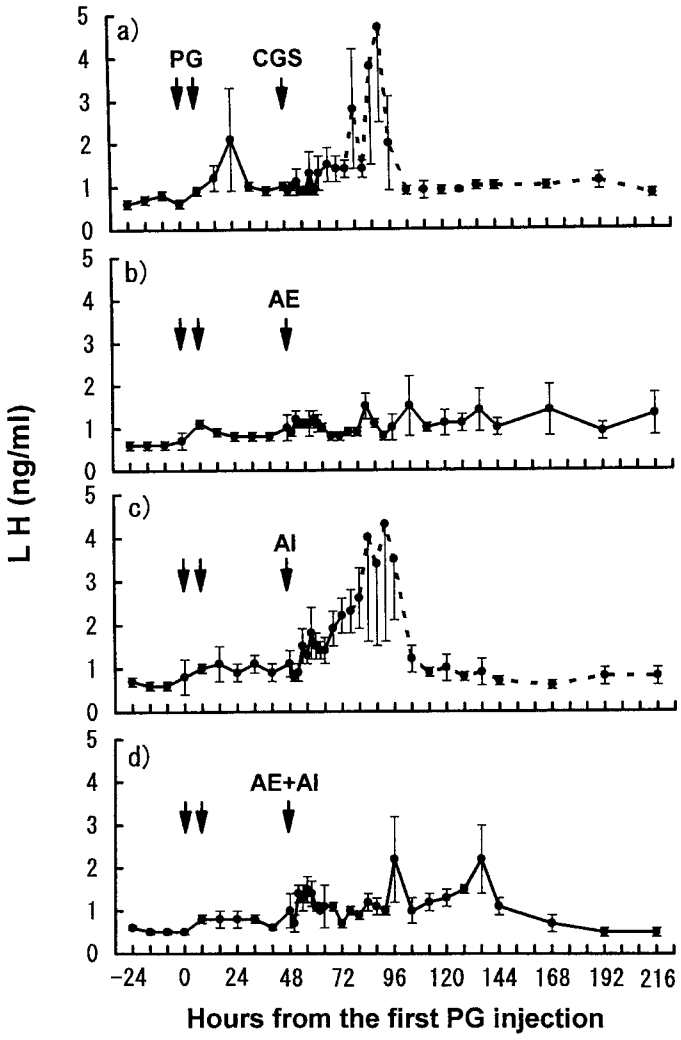

FIG. 2. Changes in plasma concentrations of LH in cows that received an i.v. bolus injection of (a) 100 ml castrated-goat serum (CGS; control serum), (b) 100 ml estradiol antiserum (AE), (c) 100 ml inhibin antiserum (AI), or (d) 100 ml estradiol antiserum and 100 ml inhibin antiserum (AE+AI) at 48 h after the first PG injection (Time 0) on Day 10 of the estrous cycle (Day 0 = day of estrus). In the control and inhibin-immunized animals, solid lines are replaced with dashed lines after the time when the first animal started an LH surge. Arrows indicate injections of PG or sera. Values are means ± SEM for four cows.

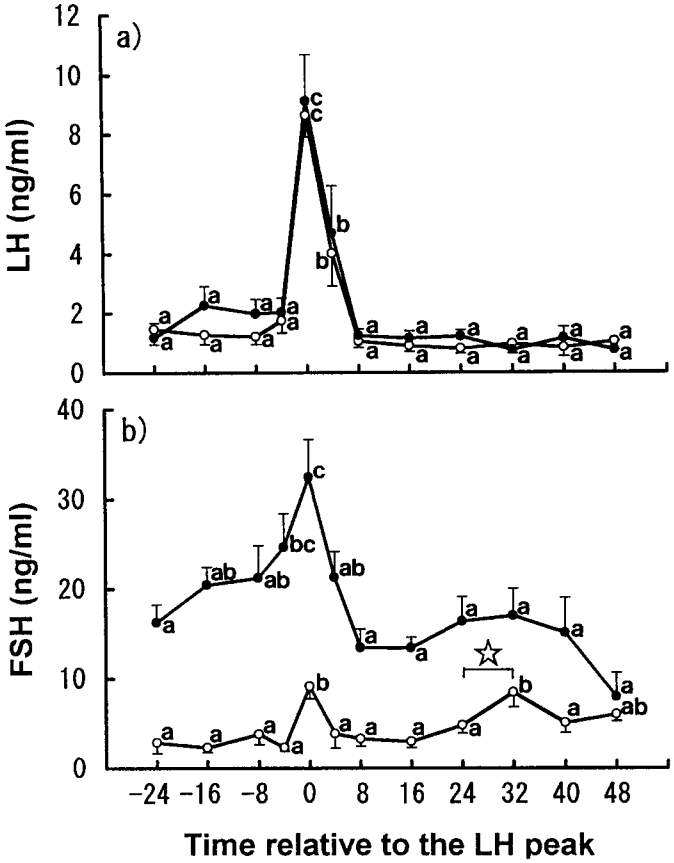

FIG. 3. Changes in plasma concentrations of (a) LH and (b) FSH around the day of estrus in control (open circles) and inhibin-immunized (solid circles) animals. The data were clustered around the peak of the increase in plasma LH on the day of estrus. Values are means ± SEM for four cows. The star signifies detection of ovulation in the controls. Means without common characters are significantly ($p < 0.05$) different in each treatment (Duncan's Multiple Range test).

immunized animals was 32.4 ± 4.2 ng/ml—much higher than in the controls (9.1 ± 1.4 ng/ml). In the combined-immunized animals, a rise in plasma FSH coincidentally with the LH increase was not observed, but a further increase in plasma concentrations of FSH was noted between 72 and 96 h (Fig. 4d). There were no significant differences in plasma levels of FSH between the inhibin- and combined-immunized groups from 48 to 92 h (Fig. 4, c and d). Concentrations of FSH in the inhibin-immunized animals decreased ($p < 0.01$) at 104 h from a maximum between 84 and 92 h and further declined to the pretreatment level at 136 h. In the combined-immunized animals, however, concentrations of FSH remained elevated until 144 h and then declined to the pretreatment level at 168 h. Between 96 and 144 h, concentrations of FSH in the combined-immunized animals were higher than in the inhibin-immunized animals.

Plasma Concentrations of Estradiol and Progesterone

There were significant increases ($p < 0.05$) in plasma concentrations of estradiol during the 48-h period after the first PG injection in all groups, and levels at the time of injection of the antisera were around 5 pg/ml (Fig. 5a). In the controls, estradiol concentrations further increased and reached a peak (11.1 ± 2.5 pg/ml) at 84 h. After a drop at 120 h, levels of plasma estradiol increased again 4 days later. Treatment with the inhibin antiserum significantly increased plasma concentrations of estradiol ($p < 0.01$) compared with the control values. Concentrations of estradiol increased over 30 pg/ml between 120 and 144 h and then declined to the pretreatment level by 192 h. Because of the presence of antibodies against estradiol in the circulation, it was not possible to measure concentrations of free plasma estradiol in the two estradiol-immunized groups.

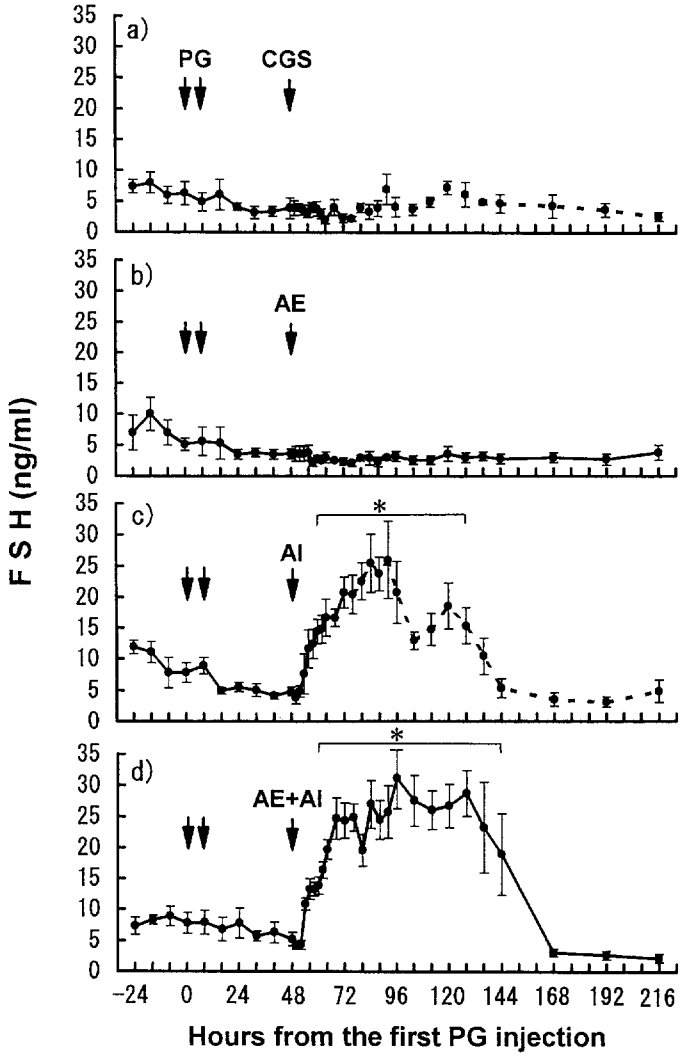

FIG. 4. Changes in plasma concentrations of FSH in cows that received an i.v. bolus injection of (a) 100 ml castrated-goat serum (CGS; control serum), (b) 100 ml estradiol antiserum (AE), (c) 100 ml inhibin antiserum (AI), or (d) 100 ml estradiol antiserum and 100 ml inhibin antiserum (AE + AI) at 48 h after the first PG injection (Time 0) on Day 10 of the estrous cycle (Day 0 = day of estrus). In the control and inhibin-immunized animals, solid lines are replaced with dashed lines after the time when the first animal started an LH surge. Arrows indicate injections of PG or sera. Values are means ± SEM for four cows. $*p < 0.05$ compared with the value for the respective control (Student's *t*-test).

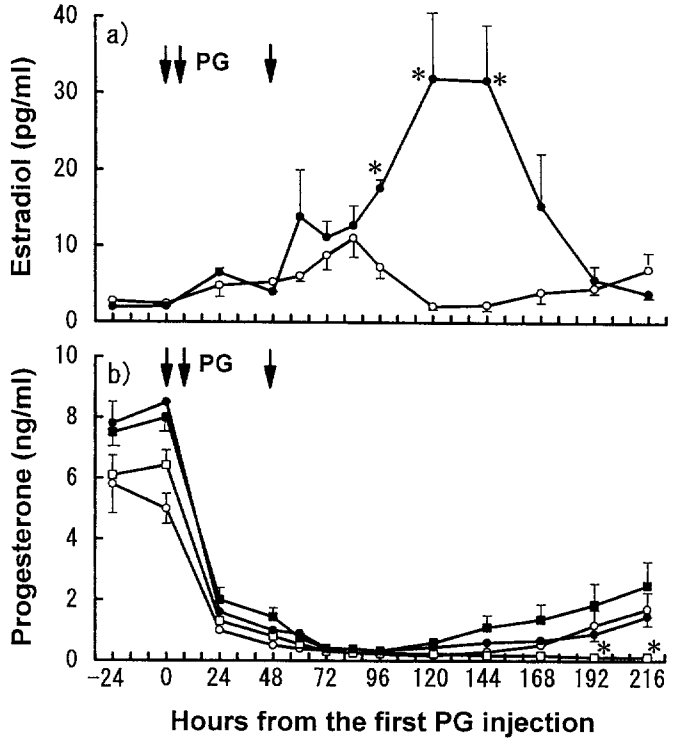

FIG. 5. a) Changes in plasma concentrations of estradiol in cows that received an i.v. bolus injection of 100 ml castrated-goat serum (CGS; control serum) (open circles) or 100 ml inhibin antiserum (AI) (solid circles). b) Changes in plasma concentrations of progesterone in cows that received an i.v. bolus injection of 100 ml castrated goat serum (CGS; control serum) (open circles), 100 ml estradiol antiserum (open squares), 100 ml inhibin antiserum (AI) (solid circles), or 100 ml estradiol antiserum and 100 ml inhibin antiserum (solid squares) (AE + AI) at 48 h after the first PG injection (Time 0) on Day 10 of the estrous cycle (Day 0 = day of estrus). Arrows indicate injections of PG or sera. Values are means ± SEM for four cows. $*p < 0.05$ compared with the value for the respective control (Student's *t*-test).

Plasma concentrations of progesterone had fallen ($p < 0.01$) by 24 h after the first PG injection in all groups and were less than 0.5 ng/ml by 120 h (Fig. 5b). Then, in the controls, concentrations of progesterone significantly ($p < 0.05$) increased, reaching 1.6 ± 0.3 ng/ml 5 days after estrus (216 h). Progesterone levels in all animals given estradiol antiserum remained low (< 0.2 ng/ml) until the end of the study, suggesting the failure of ovulation. Among the inhibin- and combined-immunized groups, one animal in each group did not show any increase in plasma concentrations of progesterone, while progesterone levels in the other animals had increased over 1 ng/ml at the end of the experiment.

Ovarian Examination

In the controls, a single large follicle (12.2 ± 0.3 mm in diameter) had ovulated by 120 h and the formation of a CL was confirmed 4 days later (216 h) (Fig. 6a). Another large follicle developed during the 4-day period after ovulation. In animals that had been given the estradiol antiserum, the largest follicle (12.3 ± 0.7 mm) at 96 h increased in size but did not ovulate (Fig. 6b) and had reached over 20 mm by the end of the experiment (216 h). No CL was observed in the ovary at 5 days after estrus. In contrast, treatment with the inhibin antiserum resulted in a marked ($p < 0.01$) increase in the numbers of follicles of various sizes as compared with those in the control group (Fig. 6c). During the first 72 h after injection of the inhibin antiserum, there was a significant increase ($p < 0.05$) in the number of small (48 h) and medium-sized follicles (72 h) as compared with the number just before immunization. An increase in the number of large follicles was seen at 120 h after administration of the inhibin antiserum, in comparison with the number just before immunization (6.3 ± 1.5 vs. 0.5 ± 0.3). After administration of the combination of the inhibin and estra-

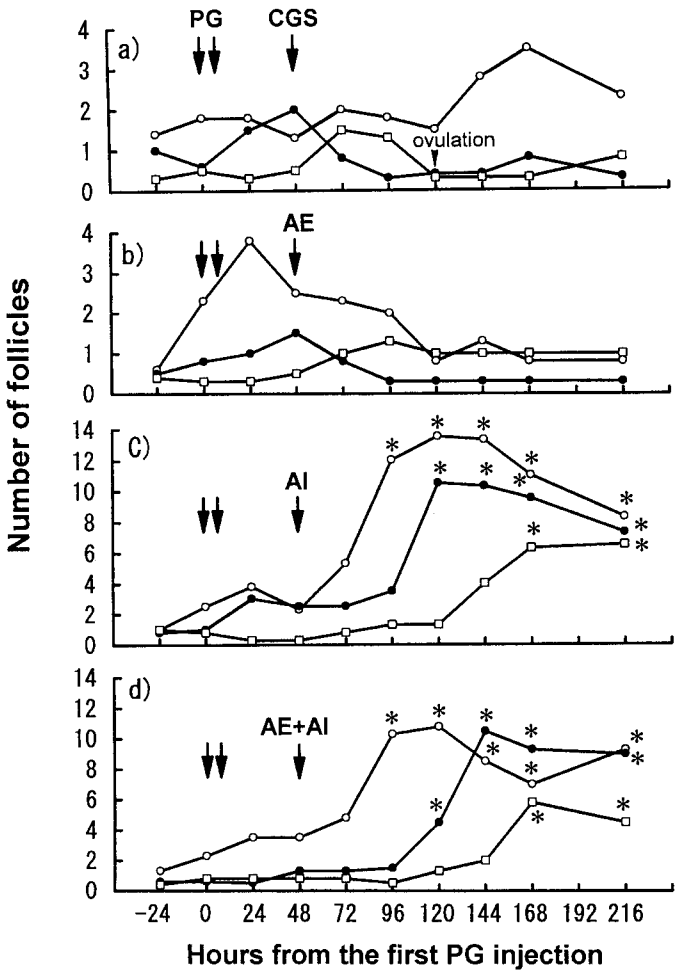

FIG. 6. Mean number of follicles in cows given an i.v. bolus injection of (a) 100 ml castrated-goat serum (CGS; control serum), (b) 100 ml estradiol antiserum (AE), (c) 100 ml inhibin antiserum (AI), or (d) 100 ml estradiol antiserum and 100 ml inhibin antiserum (AE+AI) at 48 h after the first PG injection (Time 0) on Day 10 of the estrous cycle (Day 0 = day of estrus). Small follicles (≥ 4 < 7 mm in diameter) are represented by open circles, medium follicles (≥ 7 < 10 mm) by solid circles, and large follicles (≤ 10 mm) by open squares. Arrows indicate injections of PG or sera. *$p < 0.05$ compared with the value for the respective control (Student's t-test).

diol antisera, an increase in the number of antral follicles (≥ 4 mm) was also induced; this increase was comparable to that in the inhibin-immunized animals (Fig. 6d). In the inhibin- and combined-immunized groups, ovulation(s) could not be detected by the ultrasound scanner because of the appearance of a large number of growing follicles. However, no CL was observed at 5 days after estrus.

DISCUSSION

The present study clearly demonstrates that injection of inhibin antiserum during the follicular phase resulted in a marked increase in plasma concentrations of FSH with a coincident increase in the number of antral follicles, providing clear evidence that inhibin is an important regulator for FSH secretion during this period. The present results and our previous report [19] indicate that inhibin has an important role in the regulation of FSH secretion during the bovine estrous cycle.

The control and inhibin-immunized animals showed a significant rise in plasma LH (≥ 8 ng/ml) on the day of estrus; the results indicate the occurrence of the LH surge in these animals in view of the fact that the sampling interval was 4 h during the time of the LH increase. But no animals in the two estradiol-immunized groups showed an LH surge or estrous behavior during the period of observation. This result is consistent with those from a previous study [30] showing that active immunization against estradiol interfered with the onset of the LH surge, and confirms previous evidence that estradiol is essential for the occurrence of the preovulatory LH surge in cows [31–34]. Although we cannot make assertions about pulsatile secretion of LH, the basal concentration of LH, obtained from sampling at 2- to 4-h intervals, was not affected after administration of the inhibin antiserum, confirming the hypothesis that inhibin is not involved in the regulation of basal LH secretion in cows.

Suppression of FSH concentration during the follicular phase is inversely related to the increase in plasma estradiol [1–4]. Replacement experiments indicated that a combination of estradiol and progesterone could account for the inhibitory effect from the ovary on FSH secretion [6]. During the follicular phase in cows, immunoreactive inhibin in the peripheral plasma showed a moderate increase, though this was much smaller than the increase in the concentration of estradiol [35]. Further, as far as we know, there are no published data on concentrations of dimeric inhibin in the peripheral circulation of normal cattle, because most inhibin assays cross-react with free α-subunit that is present in bovine plasma in substantial amounts [36–38]. These previous reports [1–4, 6, 35–38] question an endocrine role of inhibin in the regulation of FSH secretion during the follicular phase. However, in the present study, administration of the inhibin antiserum induced a significant rise in concentrations of FSH, indicating that inhibin plays an important role in the regulation of FSH secretion during the follicular phase when estradiol secretion is at its highest. Glencross et al. [12] also reported that active immunization against inhibin has a stimulatory effect on FSH secretion during the follicular phase.

In the present study, injection of the estradiol antiserum caused no observable changes in plasma concentrations of FSH. One probable explanation for this result is that the estradiol antiserum was not effective in completely neutralizing endogenous estradiol, as previously indicated [39]. A low concentration of free estradiol remaining in plasma may have exerted suppressive effects on FSH secretion, alone or in combination with inhibin. Martin et al. [40] demonstrated that synergistic action of physiological amounts of estradiol and inhibin could maintain normal FSH concentrations in ovariectomized ewes. While the possibility of failure of neu-

tralization cannot be excluded, we have tried to reduce the likelihood of this. Concentrations of active estradiol antiserum in the plasma remained over 2 μl/ml throughout the experiment. This suggests, taking into account the titer of the estradiol antiserum used in this study (1:4 800 000), that the plasma of estradiol-immunized animals continued to maintain a large capacity to neutralize endogenous estradiol. The LH surge was completely blocked in the two estradiol-immunized groups. The rise in FSH secretion was maintained for 48 h in cows passively immunized against inhibin during the midluteal phase, while plasma concentrations of estradiol increased to levels found in follicular phase or in estrus [19], suggesting that a physiological level of estradiol alone may exert a minor effect on FSH secretion in the absence of inhibin. From these results, it seemed likely that concentrations of FSH in animals passively immunized against estradiol during the follicular phase, when progesterone secretion is at its lowest, might not be affected because of the presence of inhibin in the circulation. On the other hand, it has been demonstrated, by immunoneutralization with specific antisera against the hormones [41–43], that FSH secretion in sheep is primarily controlled by both estradiol and inhibin. Further study is necessary to clarify the relative importance of inhibin and estradiol in the regulation of FSH secretion in cattle. Although passive immunization against estradiol alone did not affect concentrations of plasma FSH, the combined immunization produced a prolonged increase in plasma FSH as compared with the increase after inhibin immunization alone. The results suggest that estradiol is involved in the suppression of FSH secretion by enhancing the inhibitory action of inhibin on FSH secretion.

Inhibin-binding activity in plasma decreased in the same manner in the two inhibin-immunized groups; a similar result was also obtained following passive immunization against inhibin during the luteal phase in cows [19]. Evidence suggests that there is a large amount of free α subunit in the circulation of cattle [37, 38]. A significant rise in inhibin secretion resulting from an increase in the number of follicles has been noted in eCG-treated cows [11]. Both free α subunit and dimeric inhibin in the peripheral circulation are probably involved in the neutralization of the antibody against inhibin, and the change in the binding activity in plasma is responsible for the change in the concentration of FSH.

In the control animals that had an LH surge, ovulation and formation of CL occurred normally. This result was confirmed by changes in plasma concentrations of estradiol and progesterone. Previous studies indicated that active immunization against estradiol interfered with estrus and induced the formation of cystic follicles [44, 45]. In the present study also, ovulation did not occur, and the formation of a cystic follicle was noted in the estradiol-immunized animals, which did not have an LH surge; these observations are consistent with no increase in plasma progesterone levels. Passive immunization against inhibin, alone or combined with estradiol, resulted in a marked increase in the number of follicles as previously reported in cows [19] and ewes [46]. This suggests that the rise in plasma concentrations of FSH stimulates development of a new cohort of follicles. In fact, injection of the same inhibin antiserum (dosage, 75 ml) on Day 9 or 10 of the estrous cycle, followed by the injection of PG 48 h later, was found to induce a superovulatory response in Holstein heifers [47]. Although we were not successful in detecting ovulation because of the presence of large number of follicles, no CL could be observed in any of the animals in the inhibin- and combined-immunized groups. On the other hand, plasma concentrations of progesterone showed an increase in these animals. The reason for the discrepancy between ultrasonographic observation and profiles of plasma progesterone is not clear. However, there is a possibility that we failed to find the CL from the cohort of a large number of follicles, especially in the inhibin-immunized animals that had an LH surge, because the CL would have been small at the time the study ended (5 days after estrus). The formation of luteinized follicles may be responsible for the rise in plasma progesterone in the combined-immunized animals; we could not obtain information about luteinization of the granulosa layer from ultrascanning observation.

In conclusion, the present results demonstrate that inhibin has an important role in the regulation of FSH secretion during the follicular phase when estradiol secretion is its highest. The present findings, in conjunction with previously reported evidence [12, 19], indicate that ovarian inhibin has a primary role in the suppression of FSH secretion throughout the estrous cycle in the cow.

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