Changes in Plasma Concentrations of Immunoreactive Inhibin, Estradiol and FSH Associated with Follicular Waves during the Estrous Cycle of the Cow

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Abstract. Changes in plasma concentrations of immunoreactive (ir-) inhibin, steroid hormones and gonadotropins were determined in relation to follicular growth throughout the bovine estrous cycle. Three waves of follicular development occurred; the first, second and third waves were observed during the early-luteal, mid-luteal and follicular phases, respectively. A dominant follicle was identified in each wave. During the growth of the dominant follicle, the other follicles ceased their growth, while the emergence of follicular wave was observed after the dominant follicle ceased to grow or ovulated. Plasma concentrations of ir-inhibin increased (p<0.05), concomitantly with the emergence of each wave. A high concentration of ir-inhibin was noted in the growing phase of each dominant follicle. Concentrations of estradiol in the plasma increased (p<0.01) coincidentally with the growth of the first and third dominant follicles. However, during the second wave, plasma estradiol levels did not show any significant rise, suggesting that the second dominant follicle has a very low activity to produce estradiol. Progesterone levels declined (p<0.01) sharply due to the spontaneous luteolysis and increased again during the early luteal phase, reached the high levels during the mid-luteal phase. Concentrations of plasma FSH were high before the emergence of each follicular wave and began to decrease in accordance with the emergence of the wave, then remained low until the dominant follicle regressed or ovulated. These results suggest that the dominant follicle, during its growing phase, lowers the plasma FSH concentration by enhancing the secretion of inhibin alone and or estradiol, and suppresses the recruitment and growth of the other follicles. In addition, progesterone also probably affects FSH secretion during the early-luteal phase in combination with estradiol.

Key words: Inhibin, Estradiol, FSH, Follicular wave, Bovine estrous cycle.

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Ultrasonographic examination reveals that two or three waves of follicular development occur during the bovine estrous cycle. Each wave was first characterized by the development of a cohort of small follicles, then one follicle becomes dominant and the remainder regress. After the dominant follicle ovulates or ceases to grow, the next follicular wave appears [1–5]. During the
The growing phase of the first and third dominant follicles, concentrations of plasma FSH are relatively low [2, 6, 7]. Cauterization of the growing dominant follicle of the first wave, 3 or 5 days after ovulation, resulted in a significant increase in the plasma FSH concentration and advanced the emergence of the next wave [6, 8]. These results suggest the dominant follicle during the growing phase suppresses the appearance of the new wave of follicular growth by lowering plasma FSH levels. On the other hand, in vitro studies indicated that secretion of inhibin and estradiol from follicles increased coincided with follicular growth and dropped during the follicular atresia [9, 10]. Administration of inhibin [11–14] and estradiol [15–17] decreased circulating FSH levels in intact or ovariectomized heifers. From these previous reports, it seems likely that the dominant follicle, during its growing phase, exerts a suppressive effect on plasma FSH by increasing the secretion of inhibin or estradiol. However, the temporal relationships between follicular dynamics and hormonal profiles, in particular inhibin and estradiol, have not been fully clarified yet throughout the bovine estrous cycle.

In the present study, we investigated the functional change of the dominant follicle in each wave by correlating growth and regression of the dominant follicle with the changes in plasma levels of inhibin and estradiol. We also examined temporal relationships between plasma concentrations of FSH and both inhibin and estradiol.

Materials and Methods

Animals

Seven parous Japanese brown cattle (3 to 5 years old), clinically normal with regular estrous cycles, were used. Estrous behavior (standing estrus) was checked every 6 h once the decrease in the size of corpus luteum (observed by ultrasonography), suggesting regression of corpus luteum.

Ovarian examination

Ovarian follicles were examined daily during one complete estrous cycle using an ultrasound scanner (Echo Camera 210 DXII) as reported previously [2], starting 15 days after the previous estrus and ending on the day of ovulation of the subsequent cycle. Between 24 and 48 h after the onset of estrus, the ovary was examined every 2 h to detect ovulation. Growth and regression of individual follicles larger than 4 mm in diameter were identified based on successive observations. Based on the criteria of Guilbault et al. [18], follicles increased in diameter were estimated to be normal follicles and follicles ceased to increase or decreased in diameter were estimated to be regressing follicles.

Blood sampling

Blood samples were obtained by venipuncture every 6 h throughout the study, and every 2 h after the onset of estrus for 48 h in order to clarify the hormonal profiles during the periovulatory period. Plasma was removed after centrifugation and stored at -30°C until required for assays for inhibin, FSH, LH, estradiol and progesterone.

Radioimmunoassay (RIA) of inhibin

Plasma concentrations of immunoreactive (ir-) inhibin were measured by a homologous double-antibody RIA [19] using purified bovine 32 kDa inhibin for radioiodination and anti-bovine inhibin serum (TNDH-1) raised in a rabbit against a partially purified bovine inhibin. As a reference standard, purified bovine 32 kDa inhibin was used. The buffers were based on the previous report [19]. One hundred µl plasma samples, and 100 µl standards which were serially diluted with phosphate-buffered saline (50 mmol/l, pH 7.5) containing 1% (w/v) bovine serum albumin (BSA-PBS), were placed in 10 × 75 mm glass tubes. Plasma samples were made up to 200 µl with BSA-PBS, and 100 µl bovine ovariectomized serum was added to each tube of standards to compensate for the effects of serum on the RIA as previously indicated [19]. After 24 h incubation with 100 µl inhibin antiserum (at a dilution of 1:100,000) at 32°C, the iodinated bovine 32 kDa inhibin (100 µl; about 5000 cpm) was added to the each tube and then the contents were further incubated for 24 h at 32°C. Thereafter, 100 µl second antibody (goat antiserum to rabbit γ-globulin) was added and the contents were incubated for 24 h at 4°C. Following centrifugation at 1700 g for 30 min, radioactivity in the precipitate was counted. The sensitivity of the assay of inhibin, based on a 95% confidence limit of the zero standard, was 3.9 pg bovine 32 kDa inhibin/tube (39 pg/ml). The intra- and interassay coefficients of variation, calculated according to the methods of Rodbard [20], were...
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It has been reported that activin, human transforming growth factor-β (TGF-β) and a number of protein hormones show no significant cross-reactivities in this RIA system [19]. However, Knight et al. [21] demonstrated a large amount of free α subunit (26 kDa) in the bovine peripheral circulation, which shows high cross-reactivity (>100%) with several inhibin antisera [21, 22]. Also in the present study, cross-reactivity of the inhibin antiserum (TNDH-1) with bovine 26 kDa α monomer (proαC) was tested. In addition, displacement of 125I-labeled 32 kDa inhibin was examined using bovine plasma samples obtained from intact cyclic and porcine FSH treated cows. FSH treatment was carried out based on the previous report [23]. Finally, biological activity in the same plasma in which immunoreactivity was measured, was determined using an in vitro bioassay for suppression of spontaneous FSH release from cultured rat anterior pituitary cells as reported previously [24].

RIAs of FSH, LH, estradiol and progesterone

Plasma concentrations of FSH were measured by RIA [25] 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 [26] using anti-ovine LH serum (USDA-309-684P), USDA-bLH-I-1 for radioiodination and USDA-bLH-B-1 as a reference standard. The sensitivities of the assays for LH and FSH were 0.006 ng/tube (0.06 ng/ml) and 0.24 ng/tube (1.2 ng/ml) respectively, and the intra- and interassay coefficients of variation were 6.0 and 12.5 % for LH and 5.1 and 9.2 % for FSH respectively.

Plasma concentrations of estradiol and progesterone were determined as described previously [27] using antisera to estradiol-17β (GDN 244; [28] and progesterone (GDN 337; [29]). For the assay of estradiol, fat was removed from plasma samples with a solution of a mixture of 2 ml n-hexane and 0.5 ml 50% methanol (v/v) [30]. The sensitivities of the assays for estradiol and progesterone were 0.32 pg/tube (0.32 pg/ml) and 2.5 pg/tube (0.025 ng/ml) respectively. The intra- and interassay coefficients of variation were 5.4 and 9.5% for estradiol and 4.5 and 13.1 % for progesterone respectively.

Statistics

Results were subjected to analysis of variance for repeated measures [31]. 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 using the General Linear Model Procedure of the Statistical Analysis Systems [32]. A value of p<0.05 was considered to be significant.

Results

Characterization of the inhibin RIA (Figs. 1–3)

A dose-response curve obtained with bovine 26 kDa α monomer (proαC) was parallel to that obtained with bovine 32 kDa inhibin (Fig. 1). However, cross-reactivity of the inhibin antiserum (TNDH-1) with 26 kDa α monomer was about 3-fold less than that with 32 kDa inhibin (35%). Displacement curves for various plasma samples were parallel to 32 kDa inhibin standard curve, and plasma from cyclic cows were less potent than those from cows treated with porcine FSH (Fig. 2). Biological activity of inhibin in the same samples in which immunoreactivity was determined, are shown in Fig. 3. Serial dilutions of plasma from cows treated with porcine FSH showed a parallel
response to 32 kDa inhibin, whereas, plasma from the intact cows did not give a clear dose-response suppression of FSH release.

Development and regression of follicles and corpora lutea (Figs. 4 and 5)

Representative patterns of follicular development and concentrations of ir-inhibin, estradiol, progesterone and FSH were illustrated in Fig. 4. In all animals, three waves of follicular growth were identified: the first, second and third waves appeared around 2, 10 and 16 days after the LH surge, respectively. In Fig. 5, growth and regression of dominant follicles and corpora lutea, and hormonal profiles from seven animals were presented, clustered around the time of peaks of the preovulatory LH and FSH surges (0 h). Ovulatory follicle, identified retrospectively as 7 mm-sized follicle on Day -4, reached the maximum size (12.4 ± 0.4 mm, mean ± SEM) around the time of the LH surge.

Ovulation was detected from 24 to 26 h after the peak of the LH surge in all animals. The growth of the next dominant follicle (the first dominant follicle) occurred within 2 days after the detection of ovulation, followed by an progress increase in size until Day 8 (maximum diameter; 11.9 ± 0.4 mm). The second dominant follicle started its growth around Day 10. The maximum size of the second dominant follicle (9.2 ± 0.5 mm) was significantly (p<0.05) smaller than the other two dominant follicles.

Corpus luteum in the previous cycle decreased in diameter between Days -2 and 0 and was not identified on Day 1. Normal formation of corpus luteum was detected 3 days after ovulation and the diameter markedly increased thereafter, reaching over 20 mm in diameter.
Plasma concentrations of estradiol and progesterone (Figs. 4 and 5)

A distinct increase in plasma concentrations of estradiol was noted before ovulation (Day -3 to 0 and Day 19 to 21) and during the early luteal phase (Day 4 to 7) in each cow (Fig. 4). As shown in Fig. 5b, concentrations of estradiol obtained from 7 animals significantly (p<0.01) increased on Day -2 and reached the maximum (12.0 ± 0.7 pg/ml) at time of the LH peak (0 h). After a sharp drop to a nadir (<2 pg/ml) in association with the declining phase of the LH surge, plasma estradiol increased (p<0.05) again between Days 4 and 7. The rise in plasma estradiol during the early luteal phase was smaller (p<0.05) than that observed during the follicular phase. Thereafter, there were no significant changes in the concentrations of plasma estradiol.

Plasma concentrations of progesterone were high during the late luteal phase of the previous cycle, and sharply decreased in association with spontaneous luteolysis. After ovulation, plasma progesterone levels began to increase from Day 4 and reached approximately 6 ng/ml thereafter.

Plasma concentrations of ir-inhibin (Figs. 4 and 5)

In each cow, there were three instances of increases in plasma concentrations of ir-inhibin: before ovulation (Day -3 to 0 and Day 17 to 21), during the early luteal phase (Day 4 to 7) and mid-luteal phase (Day 10 to 13) (Fig. 4c). Mean concentrations of plasma ir-inhibin of seven cows increased (p<0.05) during the follicular phase and reached the maximum (405 ± 51 pg/ml) 6 h after the preovulatory LH peak, followed by a significant (p<0.05) decline at 18 h (306 ± 42 pg/ml) (Fig. 5c). In each animal, a distinct decrease in plasma ir-inhibin was noted between 12 and 24 h after the preovulatory LH peak (Fig. 4c). During the luteal phase, concentrations of ir-inhibin in the plasma

![Fig. 4](image_url)

**Fig. 4.** (a) Representative patterns of development and regression of individual follicles, changes in plasma concentrations of (b) estradiol and progesterone, (c) ir-inhibin and (d) FSH during a complete estrous cycle in a cow. Asterisks represent the detection of ovulations. The data are clustered around the time of the peaks (0 h) of the preovulatory LH and FSH surges.

![Fig. 5](image_url)

**Fig. 5.** (a) Patterns of development and regression of dominant follicles and corpora lutea, changes in plasma concentrations of (b) estradiol and progesterone, (c) ir-inhibin and (d) FSH and LH throughout the estrous cycle in 7 cows. An asterisk represents the detection of ovulation. Values are means ± SEM. The data are clustered around the time of the peaks (0 h) of the preovulatory LH and FSH surges.
showed no significant changes, although ir-inhibin levels created a small increase (p<0.1) between Days 5 and 7 (Fig. 5c).

Plasma concentrations of LH and FSH (Figs. 4 and 5)

Plasma concentrations of LH were maintained at low levels (<0.5 ng/ml) during the luteal phase, while a small increase in plasma LH over 1 ng/ml was noted after spontaneous luteolysis (Fig. 5d). A distinct rise in plasma LH occurred within 12 h after the onset of estrous behavior (standing estrus) and the peak value of the LH increase was 12.2 ± 0.8 ng/ml.

Concentrations of FSH in the plasma decreased (p<0.05) from the late luteal phase to the follicular phase (Fig. 5d). The preovulatory FSH surge occurred in accordance with the LH surge, the peak value was 21.4 ± 2.8 ng/ml. Between 18 and 30 h after the preovulatory FSH peak, a transient but apparent increase (p<0.01) in plasma FSH occurred in all animals (the second FSH surge). Concentrations of FSH then declined and remained low (<3 ng/ml) until Day 7, and increased (p<0.01) again over 7 ng/ml between Days 9 and 12. After a gradual decrease in plasma FSH on Days 13 and 14, the levels increased again from Day 15 to 17.

Relationships between dynamics of follicular waves and hormonal profiles (Fig. 6)

Since there was a difference in the timing of the emergence of each follicular wave in individual animals, data about all hormones were clustered around the initiation of each follicular wave (day 1=day of the emergence of the wave indicated by an increase in the number of follicles), to clarify relationships between follicular development and hormonal profiles (Fig. 6, b and c). Diameter of dominant follicles, and number of presumed normal follicles (≤ 4 mm) estimated by the ultrasonographic observation, were also presented (Fig. 6, a). Concentrations of plasma FSH were high prior to the emergence of each follicular wave and began to decrease (p<0.05) coincidently with the appearance of the wave, remained low during the growing phase of the dominant follicle. The number of normal follicles in each wave decreased in the period of low plasma FSH, while the dominant follicle continued to grow. Plasma concentrations of ir-inhibin increased (p<0.05) concomitantly with the emergence of each wave. In the first and third waves, concentrations of ir-inhibin and estradiol reached high levels in the growing phase of the dominant follicles and decreased (p<0.05) when the dominant follicle ceased their growth or ovulated. In contrast, plasma FSH levels showed a significant (p<0.05) increase in the regression phase or around ovulation of the dominant follicle. During the second wave, there was no significant increase in plasma concentrations of estradiol, while ir-inhibin levels increased (p<0.05). There was a clear inverse relationship between plasma concentrations of FSH and ir-inhibin throughout the estrous cycle.

Discussion

The present paper demonstrates the relationships between follicular dynamics and hormonal profiles during the complete estrous cycle of cows. Concentrations of plasma FSH were high preceded the emergence of each follicular wave and began to decrease after the emergence of the wave, remained low during the growing phase of the dominant follicle. During the period of the low plasma FSH concentration, the number of presumed normal follicles in a wave decreased, while the dominant follicle kept its growth. These results strongly suggest that the fluctuation of the circulating FSH levels is involved in the recruitment and selection of follicles. Sunderland et al. [7] demonstrated that selection of follicles and functional dominance of a follicle occurred coincidently with a decrease in plasma FSH. The present findings, together with the previous evidence [6, 8, 33], also indicate that the dominant follicle in each wave exerts a suppressive effect on follicular development by lowering the concentration of plasma FSH.

Plasma concentrations of estradiol increased concomitantly with the growth of the first and third dominant follicles, while plasma estradiol levels showed no increase during the growing phase of the second dominant follicle. Several previous studies reported the increases in plasma estradiol during both the follicular and the early luteal phases [2, 7, 34]. Ireland et al. [35] demonstrated that a single ovary was responsible for the increase in the concentration of estradiol in utero-ovarian vein plasma during these periods. The secretion of estradiol from dominant follicles or healthy follicles larger than 10 mm in diameter was much greater than the other small or atretic follicles [34, 36].
Dominant follicles are probably responsible for most of the changes in plasma estradiol during these periods. In contrast, the second dominant follicle seems to have the much lower activity to secrete estradiol compared to the other two dominant follicles. Theca cells of the second dominant follicle may produce the least aromatizable substrates due to the lowest pulse frequency of plasma LH during the mid-luteal phase [37, 38].

A significant rise in the plasma concentration of ir-inhibin was noted in each follicular wave when the data were clustered around the initiation of the wave. In the third wave, plasma concentrations of ir-inhibin reached high levels, as well as estradiol, coincidentally with the growth of a dominant follicle and decreased around ovulation of the follicle. The present results agree with previous reports that plasma ir-inhibin increased during the follicular phase and decreased after the LH surge [39, 40]. The results are also consistent with the finding in cows that inhibin-like bioactivity increased before the LH surge in utero-ovarian vein and follicular fluid of presumed ovulatory follicle [41]. In the growing phase of the first dominant follicle, a moderate high concentration of ir-inhibin was observed coincidentally with an increase in plasma estradiol, and plasma inhibin decreased when the follicle ceased its growth. Dominant fol-
 follicles in the first and third waves are likely to be important sources of the circulating inhibin, as well as estradiol. In the second wave, ir-inhibin levels showed a significant increase, whereas plasma estradiol showed no change. The previous reports suggest that granulosa cells of immature follicles which had a little aromatase activity, has the ability to produce a significant amount of inhibin [9, 10, 42]. From these findings [9, 10, 42], it seems likely that the second dominant follicle may contribute the rise in plasma ir-inhibin levels, while the activity of estradiol production of the follicle is much lower than the other two dominant follicles.

It has been demonstrated that bovine granulosa cells produce a considerable quantity of free inhibin subunit (proαC and αNαC) devoid of biological activity [21, 22, 43] and these molecules were secreted into the peripheral circulation [21]. It is recognized that proαC cross-reacts extensively with antisera against synthetic α subunit fragment [12, 21] and against native 32 kDa inhibin [22]. The antiserum used in the present RIA cross-reacted with proαC as well as 32 kDa inhibin, though the cross-reactivity was much less than 32 kDa inhibin. And a close correlation existed between plasma concentrations of inhibin measured by RIA and by bioassay. Our previous report demonstrated that inhibin immunoreactivity showed a parallel change with inhibin bioactivity in the peripheral circulation of cows induced to superovulate with equine chorionic gonadotropin [44]. The present results and the previous report [44] support a view that the RIA used in the present study measures inhibin bioactivity. However, a complete assay system to measure only dimeric inhibin is required to know the exact amount of inhibin in the circulation.

There were clear inverse relationships between concentrations of plasma FSH and ir-inhibin during all three waves. Concentrations of plasma estradiol were inversely related with plasma FSH during the first and third waves. It is well known that inhibin [11–14] and estradiol [15–17] can suppress FSH secretion in intact or ovariectomized heifers. Recent data [30] that passive immunoneutralization of inhibin during the follicular phase resulted in a significant elevation of plasma FSH, provides strong evidence that inhibin is actually involved in the inhibitory regulation of FSH secretion. From these findings, the first and third dominant follicles are likely to cause a suppression of plasma FSH levels, by increasing the secretion of inhibin and estradiol. In contrast with the other dominant follicles, the second dominant follicle seems to be involved in the suppression of plasma FSH by the enhancement of inhibin production.

Progesterone diminished pulsatile secretion of gonadotropin-releasing hormone (GnRH) [45] by lowering the activity of the hypothalamic GnRH pulse generator [46], and the suppressive effect of progesterone was enhanced by the presence of estradiol [46]. It is probable that progesterone is involved in the decline of plasma FSH during the first and second follicular waves, alone or in combination with estradiol.

In summary, the results in the present study strongly suggest that the dominant follicle in each wave suppresses plasma FSH levels by the enhancement of the secretion of inhibin alone and or estradiol, and thereby inhibits the emergence of the next wave as well as the growth of the other follicles in the same wave.

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