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Plasma Concentrations of Ir-inhibin, Inhibin A, Inhibin pro-αC, FSH, and Estradiol-17β During Estrous Cycle in Mares and Their Relationship with Follicular Growth

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The relationship among plasma levels of immunoreactive (ir)-inhibin, inhibin A, inhibin pro-αC, FSH, estradiol-17β and follicular growth were investigated during the normal estrous cycle in mares. Seven mares were used for two successive normal estrous cycles. Follicular diameters and ovulation were obtained by transrectal ultrasonography, and blood samples were collected daily by jugular venipuncture for hormonal assay. The results showed that inhibin A was inversely correlated ($r = -0.59$, $p < 0.0001$) with FSH indicating its contribution to negative feedback control of FSH secretions from the pituitary gland. Estradiol-17β increased during the follicular phase reaching a peak (37.9 ± 3.8 pg/mL) 2 d before ovulation. Estradiol-17β was positively correlated ($r = 0.78$, $p < 0.0001$) with inhibin A. The high levels of inhibin A and estradiol-17β were associated with the growth of the preovulatory dominant follicle and inversely correlated with FSH suggesting that both hormones are products of the large dominant follicles and were responsible for the decline in FSH secretion during the follicular phase of estrous cycle. In conclusion, an inverse relationship between inhibin A and FSH was clearly demonstrated indicating that inhibin A has a key role in the negative feedback control of FSH from the pituitary gland. In addition, inhibin A and estradiol-17β secretions were associated with the growth of the preovulatory dominant follicle and were positively correlated.

Key Words: Inhibin; FSH; estradiol; follicle; mare.

Introduction

The gonadal glycoprotein inhibin has been implicated as a suppressive regulator of FSH secretion from anterior pituitary gland (*1*). Inhibin consists of two disulfide-linked chains (α and either βA or βB subunits) to form inhibin A or inhibin B, which is secreted as variously processed forms, with the 32-kDa dimer the most mature and bioactive form *(2,3)*. The inhibins are primarily produced by ovarian granulosa cells, although their production is differentially regulated. In humans *(4,5)*, inhibin B is thought to be produced primarily by the gonadotropin-sensitive developing antral follicle, whereas inhibin A is thought to be produced primarily by the dominant follicle. In cattle, the predominant inhibin form found in follicular fluid is inhibin A, and inhibin B levels, as measured by ELISA, are much lower *(6)*. The understanding of the physiology of inhibin has been advanced by the development of serum or plasma assays *(7–11)*. However, most previous studies in normal cyclic mares have reported circulating concentrations of immunoreactive (ir)-inhibin *(12–14)*. These assays do not distinguish between the biologically active dimeric forms of inhibin and free monomeric α-subunits, which are not thought to be biologically active. The α-subunits are present in very high concentrations in the circulation, effectively masking the bioactive dimeric forms *(15)* and hence also our understanding of the role of the bioactive dimeric inhibin forms. Moreover, only few publications *(16,17)* concern measuring inhibin A in mares. The objective of the present study was to determine plasma levels of ir-inhibin, inhibin A, inhibin pro-αC, estradiol-17β, and FSH and their relation to follicular growth during the normal estrous cycle in mares.

Results

Relationship among Plasma Concentrations of Ir-inhibin, Inhibin A, Inhibin pro-αC, FSH, and Estradiol-17β

The relationships among plasma concentrations of ir-inhibin, inhibin A, inhibin pro-αC, FSH, and estradiol-17β

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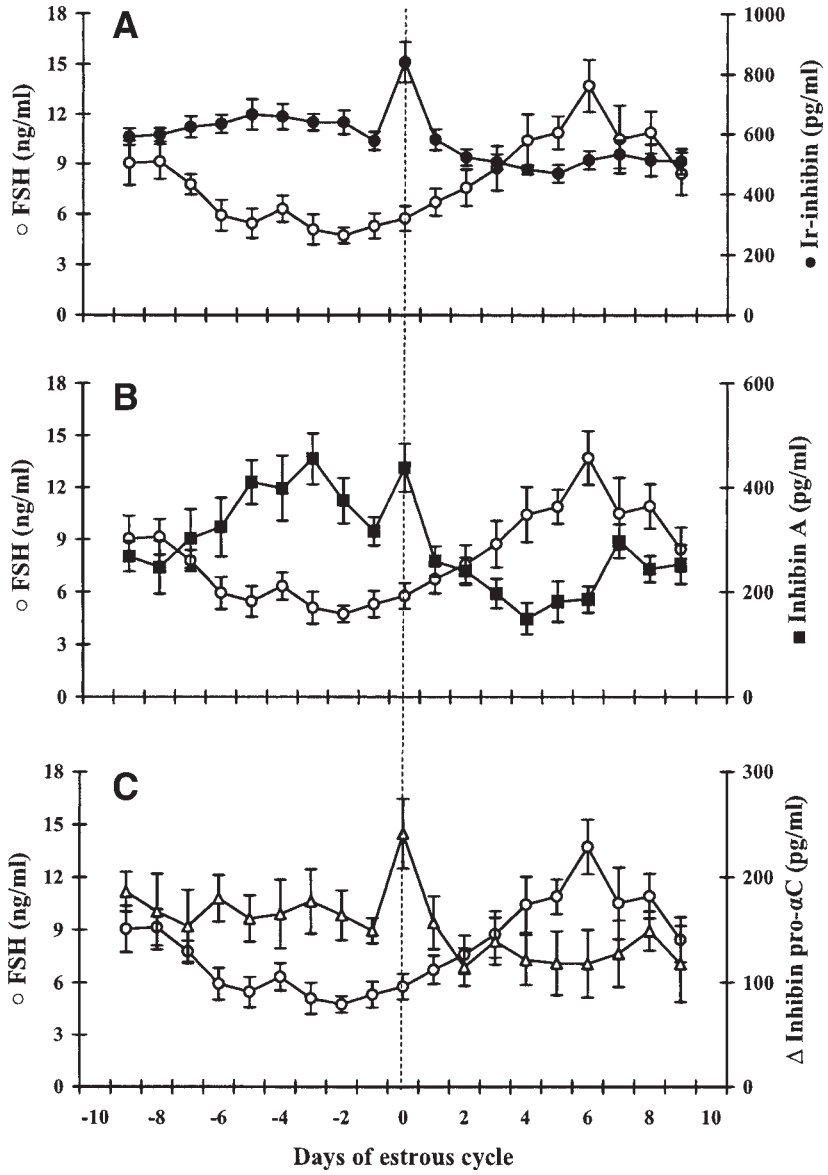

Fig. 1. Changes in plasma concentrations of FSH (-○-), immunoreactive (ir)- inhibin (**A**), inhibin A (**B**), and inhibin pro-αC (**C**) during estrous cycle in mares. Data are aligned around the day of ovulation (Day 0) and the results represent means ± SEM of seven mares for two estrous cycles. Dashed vertical line indicates the day of ovulation.

throughout the estrous cycle in mares are shown in Figs. 1 and 2. There was a nonsignificant increase in ir-inhibin during the follicular phase of estrous cycle in mares. However, there was a significant ($p < 0.05$) increase in inhibin A level during the same period. Thereafter, both ir-inhibin and inhibin pro-αC sharply increased on the day of ovulation followed by an abrupt decline. On the other hand, the mean plasma concentrations of FSH showed a significant ($p < 0.01$) increase 4 d after ovulation coincided with the decrease in inhibins concentrations, and remained high until 8 d before next ovulation (Fig. 1). The mean concentrations of estradiol-17β increased in the follicular phase reaching a peak (37.9 ± 3.8 pg/mL) 2 d before ovulation (Fig. 2). Figure 3 shows the correlations between inhibins and both FSH and estradiol-17β. There was a highly significant inverse correlation between inhibin A and FSH and a highly significant positive correlation between inhibin A and estradiol-17β.

Relationships among Plasma Concentrations of Ir-inhibin, Inhibin A, Inhibin pro-αC, FSH, Estradiol-17β, and Follicular Growth

The relationships among plasma concentrations of ir-inhibin, inhibin A, inhibin pro-αC, FSH, estradiol-17β, and follicular growth are shown in one representative mare (Figs. 4 and 5). Plasma concentrations of FSH increased sharply 4 d after ovulation and remained high until the dominant follicle reached 3 cm in diameter, then declined concomitant with increased secretions of inhibin A and estradiol-17β from the preovulatory dominant follicle. On the other

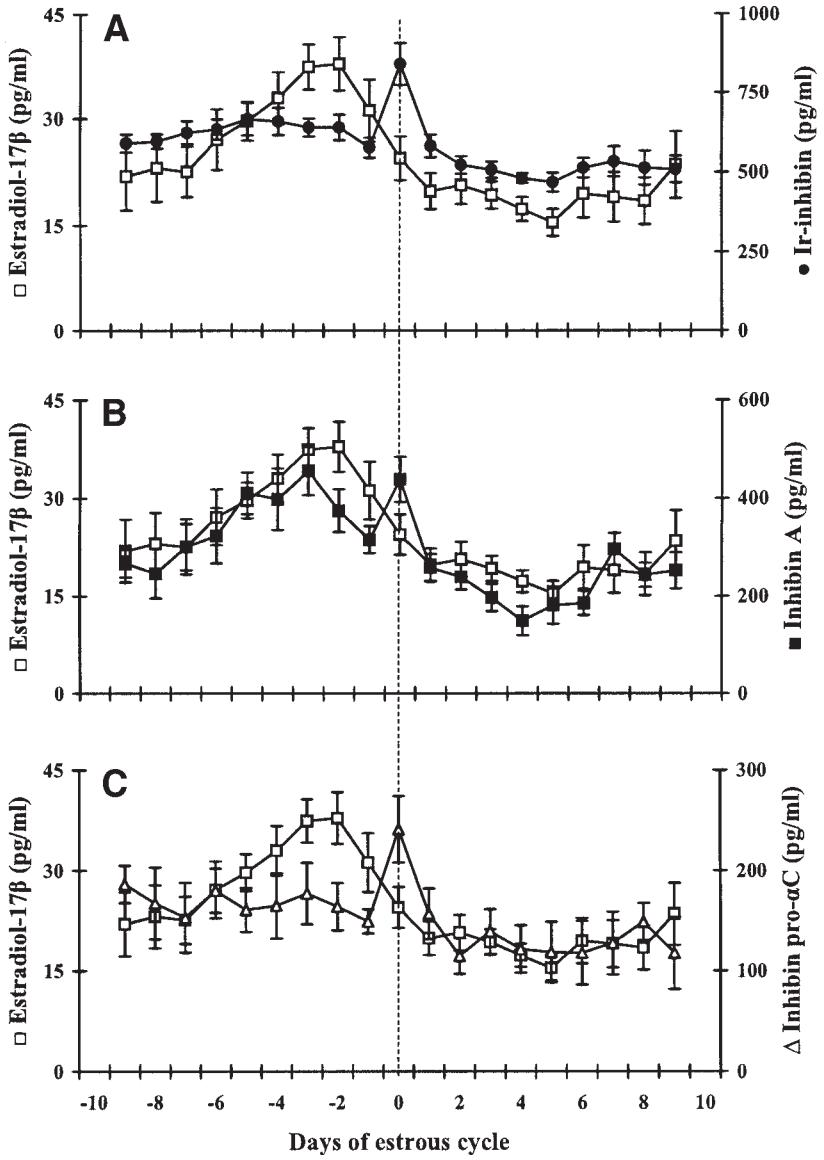

Fig. 2. Changes in plasma concentrations of estradiol-17β (-□-), immunoreactive (ir)-inhibin (**A**), inhibin A (**B**), and inhibin pro-αC (**C**) during estrous cycle in mares. Data are aligned around the day of ovulation (Day 0) and the results represent means ± SEM of seven mares for two estrous cycles. Dashed vertical line indicates the day of ovulation.

hand, plasma concentrations of ir-inhibin and inhibin pro-αC showed a limited increase compared with inhibin A during the follicular phase.

Discussion

Inhibin-A molecule consists of two protein chains (α and βA), which are covalently joined by disulfide bonds. Higher-molecular-weight precursor forms of the molecule and a pro-form of the α subunit can be found in both serum and follicular fluids. In addition, free inhibin α subunits, without the β peptide, which are not biologically active, may be found in the same fluids. Owing to the presence of these potentially cross-reacting, but inactive, compounds in the serum, a significant risk of falsely elevated values may occur. Previous studies *(12–14,18)* have reported circulating concentrations of immunoreactive inhibin in cyclic mares. Plasma concentrations of immunoreactive inhibin were higher in estrus than in diestrus and were at a maximum on the day of ovulation. The present study determined ir-inhibin, inhibin A, as well as inhibin pro-αC. Inhibin A increased sharply 8 d before ovulation coincided with the growth of the preovulatory dominant follicle. Meanwhile, ir-inhibin showed a limited increase during the same period. These findings indicate that inhibin A is secreted by the growing preovulatory dominant follicle and ir-inhibin is secreted from small and large follicles. Nagamine et al. *(14)* found that inhibin βA subunit is confined to granulosa and theca cells in large equine follicles, on the other hand, the fluid collected from small follicles contained very low concentrations of both

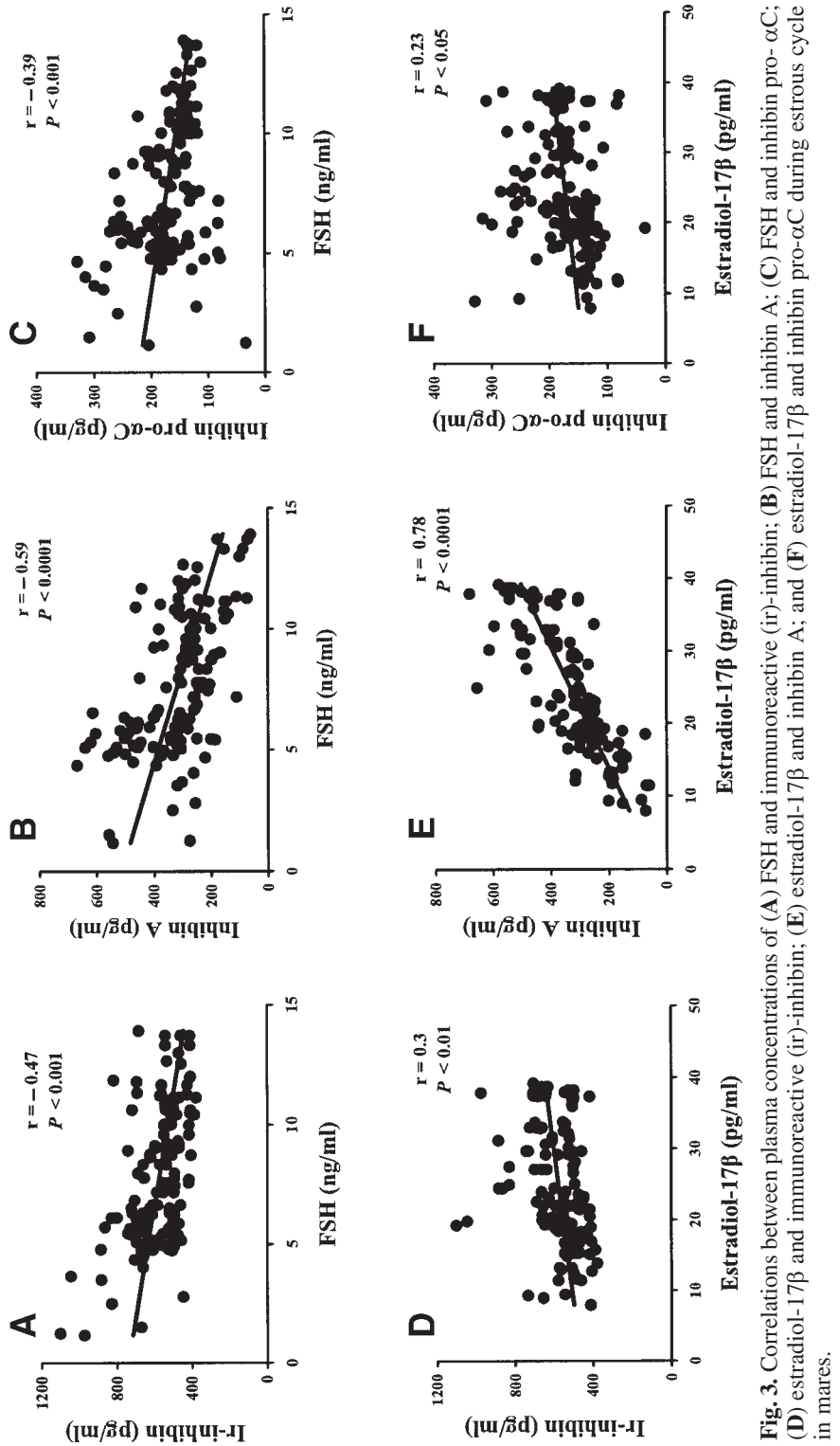

Fig. 3. Correlations between plasma concentrations of (**A**) FSH and immunoreactive (ir)-inhibin; (**B**) FSH and inhibin A; (**C**) FSH and inhibin pro- αC; (**D**) estradiol-17β and immunoreactive (ir)-inhibin; (**E**) estradiol-17β and inhibin A; and (**F**) estradiol-17β and inhibin pro-αC during estrous cycle in mares.

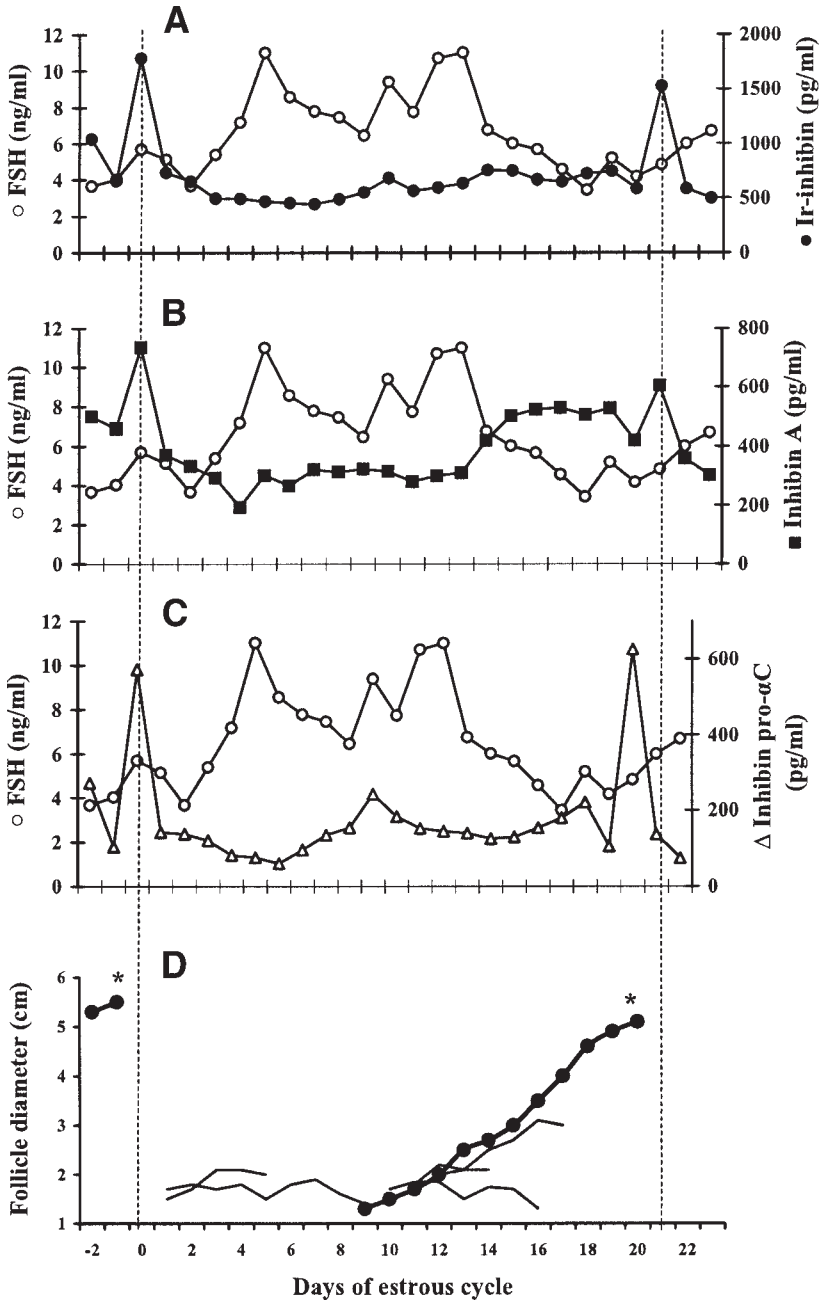

Fig. 4. Plasma concentrations of FSH (-○-), immunoreactive (ir)-inhibin (**A**), inhibin A (**B**), inhibin pro-αC (**C**), and follicular growth (**D**) during the estrous cycle in one representative mare. The dashed vertical lines indicate the day of ovulation. *indicates ovulatory follicle.

inhibin A and inhibin pro-αC, however, the fluid collected from medium sized follicle contained high concentrations of inhibin B compared with small and large follicles *(19)*. In addition, dominant follicles contain more estradiol and inhibin A than subordinate follicles in mares *(17)*. In the present study, inhibin peptides increased at the day of ovulation which was similar to the previous reports in mares *(14, 18,20)*. Increases in the levels of circulating inhibins at the day of ovulation in mares may be due to the discharge of follicular fluid. This is because the equine ovary has unusually large preovulatory follicles that are filled with a large amount of fluid rich in inhibin and estradiol-17β *(12,20)*.

The concentrations of FSH in this study decreased approx 8 d before ovulation which is similar to the previous reports in normal estrous cycle in mares *(14,21)*. An inverse relationship between plasma concentrations of ir- inhibin and FSH has been reported during the estrous cycle in mares *(12–14)*. This has been attributed to the suppressive effects of inhibin on FSH secretion *(22)*. A previous study of intravenous administration of preovulatory equine follicular fluid into ovariectomized mares, showed a significant decrease in circulating FSH levels *(23)*. In addition, both active and passive immunizations against inhibin have increased concentrations of FSH and ovulation rates in mares *(22,24)*. Our

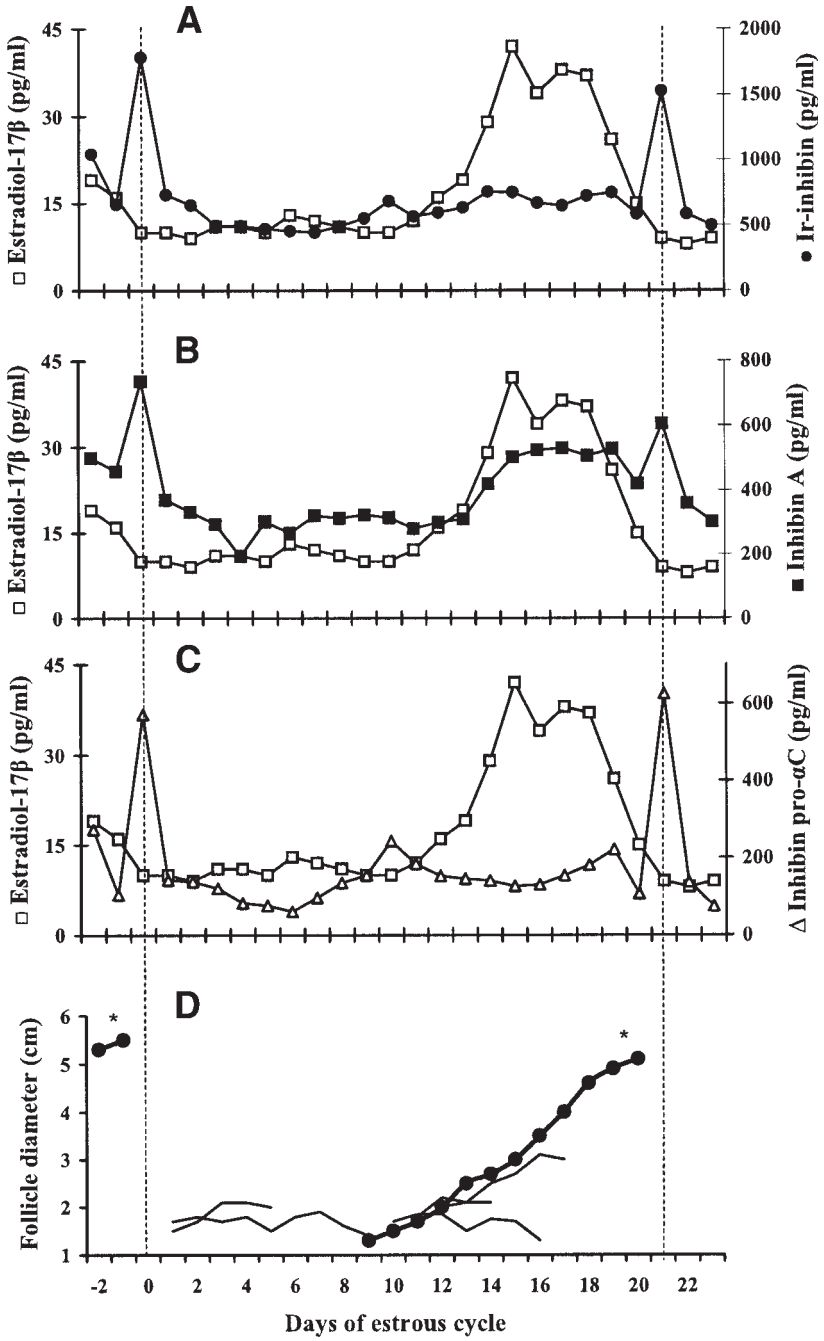

Fig. 5. Plasma concentrations of estradiol-17β (-□-), immunoreactive (ir)-inhibin (**A**), inhibin A (**B**), inhibin pro-αC (**C**), and follicular growth (**D**) during the estrous cycle in one representative mare. The dashed vertical lines indicate the day of ovulation. *indicates ovulatory follicle.

results demonstrated that the inverse relationship between FSH and inhibin A is highly significant compared with other types of inhibin indicating that inhibin A is the main regulator of FSH secretions. Additionally, estradiol-17β contributed to the regulation of FSH secretions as demonstrated by its highly significant positive correlation with inhibin A and negative correlation with FSH. Sharp et al. *(25)* reported that estradiol has a negative feedback effect on FSH in the mare and reduces the pituitary response to GnRH. In addition, the largest follicle in mares contributed to the decline in circulating FSH concentrations through increased secretion of either estradiol *(26,27)* or inhibin *(28)*. A previous study in cows *(29)* indicated that the combined effects of elevated inhibin A and estradiol account for the decline in FSH during the estrous cycle.

In summary, hormonal and follicular data of the present study demonstrated that inhibin A and estradiol-17β increased concomitant with the growth of the preovulatory dominant follicle. In addition, Inhibin A was inversely correlated to FSH suggesting its important role in the negative

feedback control of FSH from the pituitary gland. Finally, inhibin A and estradiol-17β were positively correlated.

Materials and Methods

Animals

This study was carried out on seven thoroughbred mares for two consecutive estrous cycles. Their age ranged from 9 to 17 yr and weighing 460–520 kg. These mares were clinically healthy and housed individually. They were allowed to graze together each day, and were fed twice daily on a balanced ration of pelletted feed and hay.

Ultrasound Examination and Blood Sampling

All ovarian follicles >3.0 mm in diameter and ovulation were monitored daily by ultrasound using a 5.0 MHz linear transducer. Ovulation was diagnosed by disappearance of previously recorded follicles and confirmed by detection of an area of high echogenicity (CL) in their sites. Jugular venous blood samples were collected into heparinized tubes once daily throughout the experimental period, plasma was separated after centrifugation and stored at –30°C until assayed for hormones.

RIA of Ir-inhibin, FSH, and Estradiol-17β

Concentrations of ir-inhibin in plasma were measured by double-antibody RIA systems using a rabbit antiserum against bovine 32-kDa inhibin (TNDH-1) and ^{125}I-labeled 32-kDa bovine inhibin, as described previously *(30)*. The results were expressed in terms of 32-kDa bovine inhibin. The sensitivity of the assay was 7.6 pg/tube. The intra- and interassay coefficients of variations were 7.2% and 10.5%, respectively. Concentrations of FSH in plasma were measured as reported previously *(22)*. Plasma concentrations of FSH were measured by using an antiserum against equine FSH (cat. no. AFP-2062096) provided by NIDDK. NIH, Bethesda, MD, USA. Highly purified equine FSH (cat. no. AFP-5022B) was used for radioiodination and the reference standard. The sensitivity of the FSH assay was 39 pg/tube. The intra- and interassay coefficients of variations were 8.4% and 17.2%, respectively. Concentrations of estradiol-17β in plasma were measured using ^{125}I-labeled radioligands as described previously *(31,32)*. Antisera against estradiol-17β (GDN 244) were provided by Dr. G.D. Niswender (Animal Reproduction and Biotechnology Laboratory, Colorado State University, Fort Collins, CO, USA). The sensitivity of the estradiol-17β was 0.31 pg/tube. The intra- and interassay coefficients of variations were 4.6% and 5.8%, respectively.

ELISA

Concentrations of inhibin pro-αC in plasma were measured using a two-site ELISA kit (Serotec, Oxford, UK) designed for measurement of human inhibin pro-αC. In the assay, two monoclonal antibodies directed against the pro and αC regions were used *(33)*. The amount of inhibin pro-αC was expressed in terms of the purified inhibin pro-αC from human follicular fluid. The sensitivity of the assay was 0.15 pg/tube. Intra- and interassay coefficients of variations were 3.3% and 12.4%, respectively. Concentrations of inhibin A in plasma were measured using a two-site ELISA kit. The preparation of a new monoclonal antibody to the α-subunit of cow inhibin (PPG1/14/6), together with E4 monoclonal antibody to the inhibin/activin β_A subunit, has produced an ELISA able to measure inhibin A in sheep *(34, 35)*, cows *(29)*, male equine fetus *(36)*, and goats *(37)* with a similar sensitivity to the human inhibin A ELISA. The amount of inhibin A was expressed in terms of the purified 32-kDa bovine inhibin A from bovine follicular fluid. The sensitivity of the assay was 0.76 pg/tube. Intra- and inter-assay coefficients of variation were 4.2% and 8.5%, respectively.

Statistical Analyses

The data are presented as means (± SEM). The significance of daily changes in the concentrations of each hormone during estrous cycle was analyzed by one-way ANOVA with repeated measures. The significance of difference between means was determined by Duncan's multiple-range test. Canonical correlation analysis was performed to measure correlation between inhibins and both FSH and estradiol-17β and statistical significance was obtained using Wilks' lambda test. A probability value (p) of less than 0.05 was considered to be significant. All statistical analyses were performed using the SAS computer package *(38)*.

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