Inhibin Secretion in the Mare: Localization of Inhibin \( \alpha \), \( \beta_A \), and \( \beta_B \) Subunits in the Ovary

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ABSTRACT

To determine the source of circulating inhibin and estradiol-17\( \beta \) during the estrous cycle in mares, the cellular localization of the inhibin \( \alpha \), \( \beta_A \), and \( \beta_B \) subunits and aromatase in the ovary was determined by immunohistochemistry. Concentrations of immunoreactive (ir-) inhibin, estradiol-17\( \beta \), progesterone, LH, and FSH in peripheral blood were also measured during the estrous cycle in mares.

Immunohistochemically, inhibin \( \alpha \) subunits were localized in the granulosa cells of small and large follicles and in the theca interna cells of large follicles, whereas inhibin \( \beta_A \) and \( \beta_B \) subunits were localized in the granulosa cells and in the theca interna cells of large follicles. On the other hand, aromatase was restricted to only the granulosa cells of large follicles. Plasma ir-inhibin concentrations began to increase 9 days before ovulation; they remained high until 2 days before ovulation, after which they decreased when the LH surge was initiated. Thereafter, a further sharp rise in circulating ir-inhibin concentrations occurred during the process of ovulation, followed by a second abrupt decline. After the decline, plasma concentrations of ir-inhibin remained low during the luteal phase. Plasma estradiol-17\( \beta \) concentrations followed a profile similar to that of ir-inhibin, except during ovulation, and these two hormones were positively correlated throughout the estrous cycle. Plasma FSH concentrations were inversely related to ir-inhibin and estradiol-17\( \beta \). These findings suggest that the dimeric inhibin is mainly secreted by the granulosa cells and the theca cells of large follicles; granulosa cells of small follicles may secrete inhibin \( \alpha \) subunit, and estradiol-17\( \beta \) is secreted by the granulosa cells of only large follicles in mares.

INTRODUCTION

Inhibin, a gonadal peptide hormone, is known to suppress FSH release in mammals by its direct action on gonadotrophs. It is secreted by the granulosa cells of ovarian follicles in several species, such as rats [1, 2], hamsters [3], humans [1], pigs [4], cows [4], sheep [5], and monkeys [6]; and a reciprocal relationship between circulating concentrations of inhibin and FSH during the estrous cycle in several species, such as rats [1, 2], hamsters [3], and a reciprocal relationship between circulating concentrations of inhibin and FSH during the estrous cycle. Piquette et al. [20] reported that equine granulosa-theca cell tumors have mRNA of inhibin \( \alpha \) and \( \beta_A \) subunits and proteins. However, the source of inhibin in the ovary of cyclic mares has not yet been determined.

In the present study, we used immunohistochemical and RIA techniques to determine the cellular sources and relationships of inhibin and another physiologically important ovarian hormone, estradiol-17\( \beta \).

MATERIALS AND METHODS

Animals

Thirteen clinically normal Thoroughbred mares aged 6–17 yr that were showing regular estrous cycles were used in the experiment, which ran from May to September.

Blood Sampling and Ovarian Examination

Jugular venous blood samples were collected into heparinized tubes once daily between 0900 and 1100 h throughout the estrous cycle to clarify the hormonal profiles during the examination period. The plasma was decanted after centrifugation at 1700 \( \times \) g for 10 min at 4°C and stored at −30°C until assayed for immunoreactive (ir-) inhibin, FSH, LH, estradiol-17\( \beta \), and progesterone concentrations. The reproductive tracts of the mares were examined by transrectal palpation and ultrasound scanning every 1–4 days throughout the estrous cycle.

Immunohistochemistry

Tissue fixation and preparation. Ovaries were obtained from 12 mares at the late follicular phase and the midluteal phase during the estrous cycle in the breeding season. Mares were killed by intravenous injection with a mixture of 2 g sodium thiopental (Ravonal; Tanabe Seiyaku Co., Osaka, Japan) and 500 mg suxamethonium chloride (Suxcine; Kyorin Pharmaceutical Co., Tokyo, Japan). Ovaries were separated into follicles and corpora lutea and fixed for 24 h in 4% paraformaldehyde. These follicles and corpora lutea were then dehydrated through a series of graded concentrations of ethanol and xylene, embedded in paraffin, and sectioned serially at 6 \( \mu \)m. Immunohistochemical staining for inhibin \( \alpha \), \( \beta_A \), and \( \beta_B \) subunits and aromatase was carried out as described previously [3]. For inhibin \( \alpha \) subunit, a rabbit polyclonal antibody against [Tyr30] porcine inhibin \( \alpha \) chain (1–30)NH2 (kindly provided by Dr. N. Ling, Neurocrine Bioscience, San Diego, CA), diluted 1:4000 in 50 mM PBS containing 0.55% (w:v) casein, was...
used as the primary antibody; and biotinylated goat anti-rabbit IgG and a preformed avidin-biotin-horseradish peroxidase macromolecular complex (Vector Laboratories, Burlingame, CA) were used, respectively, as second antibody and enzyme substrate. Two controls were set up in which 1) sections were exposed to normal rabbit serum (diluted 1:4000) instead of the inhibin α-chain primary antibody and 2) sections were incubated with the polyclonal inhibin α-chain antibody that had been neutralized previously with purified bovine inhibin (32 kDa). For inhibin βA and βB subunits, a mouse monoclonal antibody against synthetic inhibin βA chain (E4) [21] and a mouse monoclonal antibody against synthetic inhibin βB chain (C5) [22] (kindly provided by Dr. N.P. Groome, Oxford Brookes University, Oxford, UK), diluted 1:4000 and 1:1000, respectively, in 50 mM PBS containing 0.55% (w/v) casein, were used as the primary antibodies. Biotinylated horse anti-mouse IgG and a preformed avidin-biotin-glucosidase macromolecular complex (Vector) were used, respectively, as second antibody and enzyme substrate. A control was set up in which sections were exposed to normal mouse serum (diluted 1:4000 and 1:1000, respectively) instead of the βA and βB inhibin primary antibodies. Some sections were also stained with hematoxylin and eosin for observation of the morphology of the follicles and corpus luteum.

Sections were also stained for aromatase activity using the same technique as described for inhibin α subunit. A polyclonal rabbit antibody against human placental P450 aromatase (R-8–1; kindly provided by Dr. Y. Osawa, Medical Foundation of Buffalo, Buffalo, NY) diluted 1:1000 was used as the primary antibody.

Hormone Assays

Immunoreactive inhibin. Concentrations of ir-inhibin in the plasma samples were measured using a homologous double-antibody RIA [23]. This employed purified bovine 32-kDa inhibin for radioiodination and reference standard and an anti-bovine inhibin serum (rAS-#1989; kindly provided by Dr. D.M. de Kretser, Monash University Institute of Reproduction and Development, Clayton, Victoria, Australia) that had been raised in a rabbit against a 31-kDa fraction of bovine inhibin. The sensitivity of the assay was 7.8 pg/tube. All the samples were measured in triplicate, and the intra- and interassay coefficients of variation were 7.4% and 10.5%, respectively.

LH and FSH. Plasma concentrations of LH were measured by a heterologous double-antibody RIA using an anti-ovine LH serum (YM #18; kindly provided by Dr. Y. Mori, University of Tokyo, Tokyo, Japan) and highly purified equine LH (E98A; kindly provided by Dr. H. Papkoff, University of California, San Francisco, CA) for radioiodination and reference standard [24]. Plasma concentrations of FSH were measured in a heterologous RIA using an anti-human FSH serum raised in a rabbit (M9); Endocrine Services Limited, Warwickshire, UK) and purified equine FSH (E219B; kindly provided by Dr. H. Papkoff) for radioiodination and reference standard [24]. The sensitivity of LH and FSH RIAs was 312.5 pg/tube and 312.2 pg/tube, respectively. The intra- and interassay coefficients of variation were 8.8% and 13.0% for the LH assay and 6.9% and 9.7% for the FSH assay.

 Estradiol-17β and progesterone. Plasma concentrations of estradiol-17β and progesterone were measured by double-antibody RIAs using antisera against estradiol-17β (GDN 244 [25]) and progesterone (GDN 337 [26]; kindly provided by Dr. G.D. Niswender, Colorado State University, Fort Collins, CO) and 125I-labeled radioligands, as described previously [27]. The sensitivities of the estradiol-17β and progesterone RIAs were 0.3125 pg/tube and 1.25 pg/tube, respectively. The intra- and interassay coefficients of variation were 4.8% and 5.8% for estradiol-17β and 3.5% and 13.4% for progesterone.

Statistics

All the data are presented as means ± SEM. When there was heterogeneity of variance and the standard deviations were proportional to the means, logarithmic transformation was carried out before ANOVA. The significance of daily changes in the concentrations of each hormone during the estrous cycle was analyzed by a two-way ANOVA, with animal and day from ovulation as the two factors. Significance of difference between means was compared by Duncan’s Multiple Range test [28]. The linear coefficients of correlation (r) were calculated between the following pairs of plasma concentrations: ir-inhibin and estradiol-17β, estradiol-17β and FSH, and ir-inhibin and FSH. All differences with values of p < 0.05 were considered significant.

RESULTS

Localization of Inhibin and Aromatase in the Ovaries

In a healthy large follicle (larger than 3.0 cm in diameter), the granulosa layer consisted of 5–7 layers of cells (Fig. 1a and Fig. 2a), and the theca interna was composed of large epithelioid cells (Fig. 2a). Immunohistochemically, clearly positive staining for inhibin α, βA, and βB subunits was evident in the granulosa cells of large follicles. Moreover, positive staining for inhibin α, βA, and βB subunits was also seen in the theca cells of these large follicles (Figs. 2 and 3). In contrast, immunopositive staining for aromatase occurred only in the granulosa cells of the large follicles (Fig. 2).

In healthy small follicles (smaller than 1.0 cm in diameter), immunopositive staining for only inhibin α subunit was seen in the granulosa cells (Fig. 2). These cells did not stain for aromatase (Fig. 2) and inhibit βA and βB subunits (data not shown). The thin epithelioid theca layer did not stain for either aromatase or the three inhibin subunits (Fig. 2), nor did the luteal cells in a mature corpus luteum (data not shown). Control sections incubated with normal rabbit serum or with preabsorbed antibody showed only background staining (Fig. 1).

Hormone Changes during the Estrous Cycle

Concentrations of the pituitary gonadotropins (FSH and LH), ir-inhibin, and the steroid hormones (progesterone and estradiol-17β) in peripheral plasma all changed significantly during the estrous cycle (p < 0.05; Fig. 4), the mean ± SEM length of which was 20.3 ± 0.8 days (range 16–25 days, n = 13).

Immunoreactive inhibin. Immunoreactive inhibin levels began to increase 9 days before ovulation and remained high until 2 days before ovulation; they then declined in conjunction with the start of the LH surge. Thereafter the ir-inhibin levels increased sharply again during the process of ovulation. This ovulatory rise was followed by an abrupt decline, and the levels remained low throughout the luteal phase.

 Estradiol-17β and progesterone. Except during ovulation, plasma concentrations of estradiol-17β followed a pro-
file similar to that of ir-inhibin, and the two hormones were positively correlated throughout the estrous cycle (r = 0.20137, n = 362). Plasma concentrations of progesterone remained low (less than 1 ng/ml) during the follicular phase but began to increase after ovulation and reached a peak level 6 days later.

**FSH and LH.** Concentrations of FSH in the plasma began to increase 1 day before ovulation, reached a peak 3 days after ovulation, and began to decline again 8 days after ovulation coincidentally with the start of the increase in ir-inhibin concentrations. Plasma FSH levels remained relatively high during the rest of the luteal phase but declined again to a nadir 6 days before the next ovulation. These circulating levels of FSH were inversely related to those of ir-inhibin and estradiol-17β (ir-inhibin: r = -0.21910; estradiol-17β: r = -0.25841, n = 362).

Plasma concentrations of LH began to increase 4 days before ovulation and reached a peak 1 day after ovulation. This LH surge continued for about 7 days; plasma LH concentrations at this time were around 4 times higher than the basal levels measured 5 days before ovulation.

**DISCUSSION**

Using an established and well-controlled immunohistochemical technique, the present study demonstrated the presence of inhibin α subunit in the granulosa cells of large and small follicles, and βA and βB subunit in the granulosa cells of large follicles. The large theca interna cells of large follicles also contained ir-inhibin α, βA, and βB subunits, although the staining of α subunit was weaker than for the granulosa cells. These large luteal-like theca interna cells have been reported previously in the horse to enlarge during proestrus and attain their largest size 3 days before ovulation [29]. In the present experiment, the steroidogenic enzyme aromatase, which is an indicator of estrogen secretion, was not observed in the theca interna cells, although several investigators have suggested previously that these cells probably secrete estrogens [30, 31]. Van Niekerk et al. [29] suggested that aromatase activity might subside rapidly in the preovulatory follicle thereby triggering the sharp decline in plasma estrogen concentrations that precedes ovulation. An alternative suggestion is that the luteinized theca cells may produce androgens that are then aromatized by the granulosa cells, as a critical step in the final maturation of the follicle prior to ovulation [32, 33]. This possibility stems from the finding that the concentrations of LH receptors on the thecal cells in presumptive ovulatory follicles increase on Day 14 after ovulation, thereby providing increased responsiveness to the basal LH concentrations present at this time [33]. Moreover, since these large thecal cells undergo degeneration and do not contribute to the development of the corpus luteum [29], it has been assumed that they have a physiological role in the synthesis and/or secretion of not only inhibin, but also steroid hormones just before ovulation.

Positive staining for the inhibin α, βA, and βB subunits was not observed in luteal cells, indicating that during the luteal phase the various-sized follicles that persist in the ovaries must contribute to the basal levels of ir-inhibin in the circulation. Our results therefore suggest that the major source of dimeric inhibin in the ovaries of mares during the estrous cycle is the granulosa cells of large follicles and that the theca interna of large follicles also secretes some bioactive inhibin. In contrast, aromatase, the enzyme required to convert testosterone into estrogens, was localized only to the granulosa cells of large follicles. This result accords well with the previous finding that concentrations of estradiol-17β in the actual fluid of presumptive ovulatory follicles are at least 30- to 50-fold higher than those in the fluid of nonovulatory follicles [33]. It demonstrates con-
FIG. 2. Immunohistochemical localization of inhibin α subunit and aromatase in a large (3.5 cm in diameter) (a–c) and a small (0.5 cm in diameter) (d–f) follicle. a and d: Stained with hematoxylin and eosin; b and e: stained with the inhibin α-subunit antiserum; c and f stained with the aromatase antiserum. In the large follicle, clearly positive staining for inhibin α subunit and aromatase is seen in the granulosa cells (G) and the lutein-like thecal interna cells (T). In small follicle, positive staining for inhibin α subunit is seen only in the granulosa cells. Staining for aromatase was not observed in either the granulosa cells or the thin epithelioid theca interna cells. Bar = 50 μm.

vincingly that the granulosa cells of large follicles are the major source of estradiol-17β during the estrous cycle in mares.

Concentrations of ir-inhibin in peripheral plasma were found to be positively correlated with estradiol-17β, except during ovulation, and inversely related to FSH throughout the estrous cycle. This negative correlation between ir-inhibin and FSH was previously reported by Bergfelt et al. [18] and Roser et al. [19]. The finding suggests that the two follicular hormones, inhibin and estradiol-17β, are both inhibitory to FSH secretion in the mare. In a previous study it was observed that the administration of estradiol alone to ovariec-tomized mares induced temporary and synchronous decreases in the concentrations of both FSH and LH in peripheral blood, which were followed by an increase to above pretreatment levels, while treatment with steroid-free follicular fluid resulted in a similar decrease in FSH but not LH levels [34]. This result indicated that inhibin in follicular fluid specifically suppresses FSH secretion in the mare.

In the present study, temporary increases in plasma ir-inhibin concentrations were observed on the day of ovulation. In other mammals, such as rats [35], hamsters [36], pigs [37], monkeys [11], humans [13], and cows [38], plasma concentrations of ir-inhibin increase before ovulation and decrease when the LH surge is initiated. A similar increase in ir-inhibin levels at the day of ovulation in the mare was noted in the graphs in two previous reports [18, 19]. Although the origin of this increase is not certain, it could well be related to the very large size of the ovulatory follicle (4- to 5-cm diameter) in this species. The release of at least 30–50 ml of follicular fluid rich in inhibin into the abdominal cavity when the follicle ruptures and the absorption of these high concentrations of hormone through the peritoneum into the bloodstream may be responsible for the ovulatory peak observed. Recently, a similar temporary increase in inhibin concentrations associated with ovulation was reported in women with the use of a highly sensitive enzyme immunoassay to measure inhibin B concentrations in plasma [39]. Measurement of this temporal increase in circulating ir-inhibin concentrations may be useful in the future to determine the time of ovulation in mares.

Several investigators [40–43] have reported the prolonged LH surge associated with ovulation in the mare, and the results of the present experiment agree with these previous reports. We also found that plasma concentrations of FSH are significantly higher during the luteal phase than during the follicular phase in the mare. Similarly elevated levels of FSH during the luteal phase have been reported in cows [35], in which three small increases in plasma FSH precede the three follicular waves that occur during the es-
FIG. 3. Immunohistochemical localization of inhibin $\beta_A$ and $\beta_B$ subunits in a large (3.5 cm in diameter) follicle (a–c). a) Stained with the inhibin $\beta_A$-subunit antiserum; b) stained with the $\beta_B$-subunit antiserum; c) stained with normal mouse serum. Clearly positive staining for inhibin $\beta_A$ and $\beta_B$ subunits is seen in the granulosa cells ($G$) and the lutein-like thecal interna cells ($T$). Bar = 50 $\mu$m.

FIG. 4. Changes in plasma concentrations of a) LH, b) FSH, c) ir-inhibin, d) progesterone, and e) estradiol-17$\beta$ during the estrous cycle in Thoroughbred mares. Data are clustered around the day of ovulation, and the results represent means ± SEM of 13 mares.

trous cycle in that species. In the mare, it has been reported that two different secretory patterns of FSH may be seen with one or two peaks during the estrous cycle [40, 44, 45]. Turner et al. [46] noted that the FSH secretory pattern in female ponies tended to show two peaks in the early breeding season that declined to one peak as the season advanced. On the other hand, three peaks of FSH release were reported in the donkey [47]. In the present study, two peaks of FSH secretion were seen in most of the 13 mares that were bled daily from the first until the second ovulation after parturition. However, while some mares clearly showed the existence of one or two peaks, other mares showed indistinct FSH peaks and estrous cycles of variable...
length. Ginther [48] also suggested that misleading FSH profiles in individual mares may occur simply as a result of averaging plasma FSH concentrations. Therefore, it is necessary to analyze the individual FSH profile in relation to each follicular wave as has been demonstrated in cattle by Kaneko et al. [38].

In conclusion, the present study demonstrated that dimeric inhibin is mainly secreted by the granulosa cells of large follicles and by the theca cells of large follicles, whereas estradiol-17β is secreted by the granulosa cells of only large follicles, in the mare. It also demonstrated a temporal increase in plasma ir-inhibin concentrations in association with ovulation that may be used in the future as a measure of detecting this important event in the cycling mare.

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