A selective increase in circulating inhibin and inhibin pro-αC at the time of ovulation in the mare

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Nagaoka, Kentaro, Yasuo Nambo, Natsuko Nagamine, Shun-Ichi Nagata, Yumiko Tanaka, Hiromi Shinbo, Nobuo Tsunoda, Hiroyuki Taniyama, Gen Watanabe, Nigel P. Groome, and Kazuyoshi Taya. A selective increase in circulating inhibin and inhibin pro-αC at the time of ovulation in the mare. Am. J. Physiol. 277 (Endocrinol. Metab. 40): E870–E875, 1999.—The relationship between a selective increase in circulating immunoreactive (ir)-inhibin and the time of ovulation was investigated in mares. Concentrations of plasma ir-inhibin were measured every 4 h during the periovulatory period. Inhibin pro-αC, a precursor protein of the inhibin α-subunit, was also measured. The changes in ir-inhibin and inhibin pro-αC in circulation were parallel. Concentrations of both ir-inhibin and inhibin pro-αC in the plasma increased at the same time when ovulatory follicles ruptured, and the peak levels of circulating ir-inhibin and inhibin pro-αC were maintained for 4–8 h. There was no selective increase in plasma concentrations of estradiol-17β during the process of ovulation. These results suggest that the selective increase in ir-inhibin and inhibin pro-αC was caused by the absorption of follicular fluid after the rupture of ovulatory follicles. These results also suggest that the measuring of plasma concentrations of ir-inhibin or inhibin pro-αC in mares might be a useful method for detecting the time of ovulation.

In ovarian follicles in several mammalian species, such as rats (17, 18), hamsters (24), guinea pigs (32), pigs (27), cows (27), sheep (2), monkeys (31), and horses (20). Initially, these subunits are produced as larger precursors and then processed to produce smaller forms. Only the dimeric forms of inhibin have bioactive effects, follicle-stimulating hormone (FSH)-suppressing effects, although the α-subunit circulates in excess amounts as biologically inactive monomers.

In the present study, to define the relationship between the selective increase in circulating inhibin and ovulation in mares, peripheral concentrations of inhibin were measured during the process of ovulation by use of a specific radioimmunoassay (11). In addition, a precursor protein of inhibin α-subunit, inhibin pro-αC, was also measured using a two-site ELISA (10).

MATERIALS AND METHODS

Collection of plasma. In the first experiment, jugular venous blood samples were collected into heparinized tubes once daily from seven adult thoroughbred mares between 0800 and 1000, and blood samples were also collected. When growing follicles larger than 3.5 cm in diameter were found in ovaries, blood samples were collected every 4 h (at 0400, 0800, 1200, 1600, 2000, and 2400). During this period, ovaries were examined by transrectal palpation and ultrasound scanning every 4 h (at 0800, 1600, and 2400) to detect the ovulation. After ovulation occurred, the 4-h sampling was continued for the next 24 h. Blood samples were centrifuged at 1,700 g and 4°C for 10 min, and plasma was collected and stored at −30°C until assayed for ir-inhibin, FSH, LH, estradiol-17β, progesterone, and inhibin pro-αC concentrations.

HORSES ARE TYPICAL SEASONAL BREEDERS. Mares repeat ovulation from spring to summer in the northern hemisphere. There are many unique phenomena in horse reproduction. For example, mares show estrous behavior for 1 wk, and ovulation occurs 2 days before the end of the estrus. It is not easy to detect the most suitable time for mating a mare with a stallion, as there are no valid methods by use of endocrine signs. Although one possibility for detecting the time of ovulation is measuring the preovulatory luteinizing hormone (LH) surge, it is not as valid a method in mares as in other mammals, because the duration of the LH surge is almost 1 wk, and the peak level of LH surge is only 4–5 times higher than the basal level, indicating that the LH surge is not a suitable sign for detecting ovulation in mares (5, 7, 20, 37). On the other hand, our previous report indicated that circulating immunoreactive (ir)-inhibin increases selectively at the day of ovulation (20, 28).

Inhibins are heterodimers consisting of disulfide-linked α- and either βA- or βB-subunits (1). Inhibins are generally produced by granulosa cells of ovarian follicles in several mammalian species, such as rats (17, 18), hamsters (24), guinea pigs (32), pigs (27), cows (27), sheep (2), monkeys (31), and horses (20). Initially, these subunits are produced as larger precursors and then processed to produce smaller forms. Only the dimeric forms of inhibin have bioactive effects, follicle-stimulating hormone (FSH)-suppressing effects, although the α-subunit circulates in excess amounts as biologically inactive monomers.

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Radioimmunoassay of ir-inhibin, FSH, LH, estradiol-17β, and progesterone. Concentrations of ir-inhibin in plasma were measured using a rabbit antiserum against bovine 32-kDa inhibin (TNDH-1) and 125I-labeled 32-kDa bovine inhibin, as described previously (11). The results were expressed in terms of 32-kDa bovine inhibin. The sensitivity of the assay was 7.8 pg/tube (78 pg/ml). The intra- and interassay coefficients of variation were 7.4 and 10.5%, respectively. The anti-inhibin serum (TNDH-1) used in the present study does not cross-react with equine chorionic gonadotropin, activin, and human transforming growth factor-β (11).

Concentrations of FSH in plasma were measured using a rabbit antiserum against human FSH (no. 6, provided by the National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD). Highly purified equine FSH (provided by Dr. J. Roser, Department of Animal Science, University of California, Davis, CA and Dr. H. Papkoff, Hormone Research Laboratory, University of California, San Francisco, CA) was used as a standard and for iodination. Plasma LH was measured using a rabbit antiserum against ovine LH (YM no. 18, provided by Dr. Y. Mori, Laboratory of Veterinary Ethology, University of Tokyo, Tokyo, Japan). Highly purified equine LH was provided by Dr. J. Roser and Dr. H. Papkoff. The sensitivity of LH and FSH assays was 31.2 pg/tube (312 pg/ml) and 312.5 pg/tube (1,560 pg/ml), respectively. The intra- and interassay coefficients of variation were 6.9 and 9.7% for the FSH assay and 8.8 and 13% for the LH assay, respectively.

Concentrations of estradiol-17β and progesterone in plasma were measured by the double-antibody RIA systems by use of 125I-labeled radioligands, as described previously (21, 35). Antisera against estradiol-17β (GDN 244) (14) and progesterone (GDN 337) (6) were kindly provided by Dr. G. D. Niswender (Animal Reproduction and Biotechnology Laboratory, Colorado State University, Fort Collins, CO). The sensitivity of estradiol-17β and progesterone assays was 0.31 pg/tube (0.62 pg/ml) and 1.25 pg/tube (12.5 pg/ml), respectively. The intra- and interassay coefficients of variation were 4.8 and 5.8% for estradiol-17β assay and 3.5 and 13.4% for the progesterone assay, respectively.

Two-site ELISA for inhibin pro-αC. Concentrations of inhibin pro-αC in plasma were measured using the ELISA kits (Serotec, Oxford, UK) for the measurement of human inhibin pro-αC without modifications. In the assay, two monoclonal antibodies against the pro and αC regions were used (10). Serial dilutions of pooled equine follicular fluid (eFF) and plasma of mares were assayed to test for parallelism.

Statistical analyses of data. All data were presented as means ± SE. The significance of daily changes in concentrations of each hormone during the estrous cycle was analyzed by a two-way ANOVA, with the animal and the time from ovulation as the two factors. Significance of difference between means was compared by Duncan's multiple range test (33). The linear coefficients of correlation (r) were calculated between the following pairs of plasma concentrations: ir-inhibin and FSH, and estradiol-17β and progesterone. All differences with a value of P < 0.05 were considered significant.

RESULTS

Characterization of the inhibin pro-αC ELISA system. Serial dilutions of eFF (0.03–3.9 nl) and peripheral plasma (1.56–50 µl) of mares produced excellent dose-response curves. These curves were parallel with
the human inhibin pro-αC standard curve, indicating that it is possible to measure the concentration of inhibin pro-αC in plasma and eFF of mares using the present ELISA method (Fig. 1).

Hormonal changes during the estrous cycle. The mean concentrations of ir-inhibin, LH, FSH, estradiol-17β, and progesterone throughout the estrous cycle are shown in Fig. 2. The follicular phase and luteal phase were characterized by the concentrations of estradiol-17β and progesterone (Fig. 2C). Plasma concentrations of ir-inhibin increased slowly in the follicular phase and then decreased until just before ovulation. Thereafter, there was a selective increase in plasma concentrations of ir-inhibin on the day of ovulation (Fig. 2A). Concentrations of FSH in the plasma were high during the luteal phase compared with the follicular phase. Changes in plasma concentrations of FSH was inversely correlated to that of ir-inhibin (r = −0.297, n = 196, P < 0.05). Concentrations of LH in the plasma began to rise 3 days before the ovulation and reached a peak on the day after ovulation. The LH surge started to increase slowly, and the peak value of the LH surge was ~4–5 times higher than the basal levels (Fig. 2B). Changes in circulating estradiol-17β and progesterone showed a clear inverse relationship throughout the estrous cycle (r = −0.406, n = 196, P < 0.05). Plasma concentrations of estradiol-17β increased abruptly during the late follicular phase and reached the peak at 2 or 3 days before ovulation, followed by a gradual decline when the LH surge started (Fig. 2C).

The periovulatory changes in plasma concentrations of ir-inhibin and inhibin pro-αC are shown in Fig. 3. Changes in circulating inhibin pro-αC were parallel with those of ir-inhibin. Both circulating ir-inhibin and inhibin pro-αC showed a selective increase at the day of ovulation, indicating that there is a relationship between ovulation and a selective increase in circulating ir-inhibin and inhibin pro-αC.

Hormonal changes around the ovulation. Individual changes in the concentrations of LH, FSH, ir-inhibin, inhibin pro-αC, estradiol-17β, and progesterone in the plasma around the time of ovulation are shown in three mares (mares A, B, and C) in Figs. 4, 5, and 6. The selective increase in plasma concentrations of ir-inhibin and inhibin pro-αC was observed when ovulatory follicles were ruptured, whereas concentrations of estradiol-17β in plasma showed an abrupt decline but not a selective increase. Changes in circulating inhibin pro-αC were completely parallel with those of ir-inhibin during the periovulatory period in these mares. Plasma concentrations of FSH increased, parallel with LH, before ovulation and declined when the postovulatory increase in ir-inhibin and inhibin pro-αC occurred. The peak of ir-inhibin and inhibin pro-αC was observed at the same time when the ovulation was confirmed by

Fig. 3. Changes in plasma concentrations of ir-inhibin (○, A) and inhibin pro-αC (●, B) around the day of ovulation in mares. Each value represents mean ± SE of 14 observations. Dotted vertical lines represent day of ovulation (day 0 of estrous cycle).

Fig. 4. Changes in plasma concentrations of LH (●), FSH (○), A, ir-inhibin (□) and inhibin pro-αC (■), B, and estradiol-17β (△) and progesterone (▲), C, around ovulation during estrous cycle in mare A. Shadow area represents sampling of 4-h interval, and open area represents sampling of 24-h interval. Ovulation occurred in the hatched area.
transrectal palpation and ultrasound scanning, and the increase of circulating ir-inhibin and inhibin pro-\(\alpha\)C was observed for 8–12 h, followed by an abrupt decline. Plasma concentrations of progesterone began to increase at 3 days after ovulation.

**DISCUSSION**

The present study clearly demonstrates that circulating levels of ir-inhibin and inhibin pro-\(\alpha\)C increase selectively when ovulatory follicles rupture. The peak levels of both ir-inhibin and inhibin pro-\(\alpha\)C were detected at the same time, and the peak levels of both inhibins were maintained for 8–12 h after ovulation. The selective increase in circulating ir-inhibin and inhibin pro-\(\alpha\)C demonstrated in the present study is a specific phenomenon in mares. Previous reports in other mammals, such as rats (36), guinea pigs (32), cows (13), goats (19), pigs (12), monkeys (22), and humans (16), have shown that plasma levels of ir-inhibin increase during the follicular phase and abruptly decline when the LH surge is initiated, followed by low levels during the process of ovulation. Although exact mechanisms responsible for the selective increase in circulating ir-inhibin and inhibin pro-\(\alpha\)C during the process of ovulation in mares may be the size of ovulatory follicles. The size of mature follicles is extremely large compared with the other animals. The size of fully mature ovulatory follicles in mares reaches >6 cm in diameter. In comparison, the size of mature ovulatory follicles in cows is ~1.5–2.0 cm in diameter (13). This indicates that the volume of follicular fluid in mature follicles of mares is much larger than that in mature follicles of cows. In addition, large preovulatory follicles of mares contain a large amount of ir-inhibin, inhibin pro-\(\alpha\)C, and estradiol-17\(\beta\) in follicular fluid, and the amount of hormones was significantly greater than in follicular fluid from small and medium-sized follicles (34). These observations indicate that the preovulatory follicle contains the large volume and high concentrations of ir-inhibin, inhibin pro-\(\alpha\)C, and estradiol-17\(\beta\). It was supposed that large preovulatory follicles rupture and release follicular fluids into the peritoneal cavity. Ir-inhibin and inhibin pro-\(\alpha\)C may be absorbed immediately into the circulation, which would induce a temporary increase in circulating levels of ir-inhibin and inhibin pro-\(\alpha\)C. On the other hand, another follicular hormone, estradiol-17\(\beta\), which is a fat-soluble steroid hormone, may be absorbed into the fat of the peritoneal cavity rather than into the circulation. This may be one of the reasons that the temporary increase in circulating estradiol-17\(\beta\) was not observed at the time of ovulation.
In the present study, we used the human inhibin pro-α C ELISA assay kit to measure the plasma concentrations of inhibin pro-α C in mares. It was already reported that this assay kit was useful for measuring inhibin pro-α C in hamsters (23). Inhibin A and Inhibin B ELISA assay kits were also developed and used to measure inhibin A and inhibin B in humans (8, 9, 25), rats (3), and hamsters (23). However, such kits were not able to measure circulating concentrations of inhibin A and inhibin B in mares, whereas concentrations of these hormones in follicular fluid were detectable, probably due to low cross-reaction (34). It was reported that free forms of the α-subunit, including particularly inhibin pro-α C, were also found abundantly in serum and follicular fluids in humans (15, 26, 29). Previous papers indicated that free forms of the α-subunit might have some role in the endocrine, autocrine, or paracrine systems. Furthermore, other evidence exists that immunization of sheep with inhibin α N region impaired fertility (4) and that pro-α N-α C modulates the binding of FSH to its receptor (30).

In summary, the present study is the first to demonstrate that a selective increase in circulating inhibin and inhibin pro-α C occurs during the process of ovulation in mares. This unique phenomenon, an ovulatory surge of inhibin, may be a useful method for detecting the time of ovulation in mares.

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REFERENCES


