Peripheral concentrations of inhibin A, ovarian steroids, and gonadotropins associated with follicular development throughout the estrous cycle of the sow

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Abstract

We investigated changes in peripheral concentrations of inhibin A, total inhibin, steroids, and gonadotropins throughout the intact estrous cycle of the sow in relation to ovarian changes determined by daily transrectal ultrasonography. All visible follicles of 3 mm or more in diameter were classified as small (≥ 3 and < 6 mm) or large (≥ 6 mm). Follicular recruitment was identified in two periods of the cycle: one from the late luteal to the follicular phase, characterized by an increase in the number of small follicles followed by the appearance of large follicles; and another during the early luteal phase, consisting only of increased numbers of small follicles. Plasma concentrations of inhibin A increased ($P < 0.05$), coinciding with the two periods of follicle emergence. Estradiol (E_2) levels increased ($P < 0.05$) during the follicular phase, but not during the early luteal phase. An inverse relationship ($P < 0.01$) between the patterns of inhibin and FSH concentrations was noted around the two periods of follicle emergence, but there was no relationship ($P \geq 0.1$) between the patterns of plasma E_2 and FSH during the early luteal phase. In conclusion, measurement of plasma inhibin A levels combined with ultrasonographic examination of the ovaries revealed two periods of synchronous follicular growth during the sow's estrous cycle. The results strongly suggest that inhibin A functions as a negative feedback regulator of FSH secretion throughout the estrous cycle, whereas E_2 appears to influence FSH secretion only during the follicular phase.

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Introduction

Combined analyses of hormonal profiles and ovarian cycles in the sow's estrous cycle can provide us with basic knowledge with which we can manipulate follicular growth to improve porcine reproductive efficiency. Earlier studies based on morphological analyses of follicular populations have suggested that continuous growth and regression of follicles smaller than 6 mm in diameter occur during the porcine estrous cycle, except in the follicular phase, when several 'co-dominant' follicles are selected and grow synchronously (Clark *et al.* 1975, Kelly *et al.* 1988a). The results of an ultrasound scanning study of follicular growth in the pig ovary support this concept (Ryan *et al.* 1994). However, classification of each follicle by the incidence of granulosa cell apoptosis and by follicular estradiol (E_2) concentration suggests the occurrence of synchronous growth and regression of small follicles during the early luteal phase (Guthrie *et al.* 1995a, 1995b).

Moreover, circulating total inhibin levels show small but clear peaks during the early luteal phase of the sow's estrous cycle (Hasegawa *et al.* 1988), suggesting the existence of coordinated recruitment and regression of follicles. Characterization of follicular growth in the porcine estrous cycle therefore remains open to dispute.

Endocrine events in the peripheral circulation have been studied during the porcine estrous cycle. These studies include analyses of changes in the concentrations of gonadotropins (van de Wiel *et al.* 1981, Hasegawa *et al.* 1988, Kelly *et al.* 1988b, Guthrie & Bolt 1990, Knox *et al.* 2003), ovarian steroids (van de Wiel *et al.* 1981, Hasegawa *et al.* 1988, Knox *et al.* 2003), and total inhibin (Hasegawa *et al.* 1988, Mukai *et al.* 1989, Trout *et al.* 1992, Knox *et al.* 2003). However, the temporal relationships between follicular dynamics and hormonal profiles throughout the estrous cycle have not yet been clarified in the pig, although such combined analyses have given us new knowledge about the control of follicular growth in sheep (Ginther *et al.* 1995),

cattle (Adams *et al.* 1992, Bleach *et al.* 2001, Kaneko *et al.* 2002a, Todoroki *et al.* 2004), and goats (Medan *et al.* 2003). Moreover, interpretation of the inhibin profiles obtained by the inhibin immunoassays used in the above studies (Hasegawa *et al.* 1988, Mukai *et al.* 1989, Trout *et al.* 1992, Knox *et al.* 2003) involves uncertainty. The ovary synthesizes inhibin dimers (inhibin A, a dimer of α and β_A subunits; and inhibin B, a dimer of α and β_B) that have FSH-suppressing activity (Ling *et al.* 1985, Miyamoto *et al.* 1985, Rivier *et al.* 1985, Robertson *et al.* 1985, Fukuda *et al.* 1986); it also produces substantial amounts of free α subunits of inhibin that have no FSH-suppressing activity (Knight *et al.* 1989, Robertson *et al.* 1989, Sugino *et al.* 1989). However, because the assays used in previous studies were based on antisera directed against the α subunits of inhibin (Hasegawa *et al.* 1988, Mukai *et al.* 1989, Trout *et al.* 1992, Knox *et al.* 2003), they did not discriminate the dimeric inhibins from the free α subunits. To overcome this major limitation in inhibin assays, we have developed a sandwich-type time-resolved immunofluorometric assay (Tr-IFMA) for dimeric inhibins, which uses a combination of α and β_A or β_B antibodies (Hasegawa *et al.* 1997, Kaneko *et al.* 2002a, 2003). Tr-IFMA has proven to be applicable to the measurement of inhibin A or/and B in humans (Hasegawa *et al.* 1997), rats (Hasegawa *et al.* 1997), cattle (Kaneko *et al.* 2002a, 2003, 2006, Todoroki *et al.* 2004), and boars (Ohnuma *et al.* 2007). Other groups have quantified dimeric inhibins in the circulation of humans (Groome *et al.* 1994, 1996), rats (Fahy *et al.* 1995, Woodruff *et al.* 1996), cattle (Bleach *et al.* 2001), and sheep (Knight *et al.* 1998) with sandwich-type ELISAs. Our present study was designed to 1) examine whether Tr-IFMAs for dimeric inhibins are applicable to female pigs and, if so, 2) clarify the changes in the levels of dimeric inhibins in the circulation throughout the porcine estrous cycle in relation to follicular growth and the profiles of FSH and E_2 .

Results

Validation of time-resolved fluoroimmunoassay and Tr-IFMA of porcine inhibin

After fractionation of porcine follicular fluid (pFF) by a combination of immunoaffinity chromatography and SDS-PAGE, fluoroimmunoassay (FIA) for total inhibin detected high immunoreactivity, with peak molecular mass values of 30, 50, 72, and 108 kDa (Fig. 1a). Inhibin A IFMA also recognized, but more specifically, four high immunoreactivity peaks of the same molecular weights (Fig. 1b). Inhibin B IFMA likewise detected peaks corresponding to the same molecular weight, but each peak value was approximately one-tenth of that determined by inhibin A IFMA (Fig. 1c).

Figure 1 Molecular weight distributions of (a) total inhibin, (b) inhibin A, and (c) inhibin B in eluates from gel slices after fractionation of pFF by immunoaffinity chromatography and SDS-PAGE. Molecular weights and positions of marker proteins used to calibrate the gels are denoted at the top.

Validation of ultrasonic measurements of follicular diameters

There was a linear relationship between the diameter of follicles measured by ultrasonography (x) and the diameter of the same follicles after dissection from the ovary (y). The regression was $y = 0.97x + 0.79$ ($r = 0.975$, $P < 0.01$).

Growth and regression of follicles and corpora lutea during the estrous cycle

Mean diameter of the largest corpora lutea (CLs) was smaller on days -6 and -5 than on day -10 , and CLs were not identified from days -4 to 1 (Fig. 2a). On day 2 , formation of CLs was observed in all sows, and the CL diameter markedly increased thereafter. Each sow showed increases in the total number of follicles (≥ 3 mm in diameter) between the late luteal and the follicular phases and in the early luteal phase (Supplementary Figure 1, see section on supplementary data given at end of this article). The mean number of

Figure 2 Development and regression of maximum-sized corpora lutea (CLs) (a), and (b) changes in the numbers of small (closed circles), large (open squares), and all (open circles) follicles during the estrous cycle of pigs. Values are means $\pm$ s.e.m. ($n=8$). Daily ultrasonographic data are aligned relative to detection of ovulation on the basis of 6 h interval scanning (ovulation=hour 0).

small follicles (≥ 3 and < 6 mm in diameter) obtained from eight sows was significantly greater ($P<0.05$) on day -5 (26.3 ± 2.4 , mean $\pm$ s.e.m.) than on day -10 (14.3 ± 1.7); on day -4, it was approximately the same as on day -5. By day -2, there were significantly ($P<0.05$) fewer small follicles (16.1 ± 1.9) than on day -5, and the number had further declined by day 0 (5.9 ± 0.9) (Fig. 2b). By day 4, the number of small follicles (12.8 ± 2.0) was significantly ($P<0.05$) greater than on day 0, but it showed no significant changes thereafter. Large follicles (≥ 6 mm in diameter) were rare between days -10 and -6, but between days -4 and -1 their numbers were significantly ($P<0.05$) greater than on day -6 (0.9 ± 0.5). After an abrupt decrease from days -1 (13.6 ± 1.1) to 0 (0.3 ± 0.2), indicating ovulation, there was no increase in the number of large follicles. The pattern of change in the total number of follicles was similar to that of small follicles on all days, except between days -3 and -1, when many large follicles appeared.

Plasma concentrations of dimeric inhibins and total inhibin

In each sow, plasma concentrations of inhibin A and total inhibin showed a transient increase in the early luteal phase in addition to an increase during the follicular phase (Supplementary Figure 1), although the duration of the rises varied. The mean concentration of inhibin A was significantly greater ($P<0.05$) on day -7 (141.6 ± 11.2 pg/ml) than on day -10 (72.4 ± 12.0 pg/ml) and

remained >110 pg/ml until day -3 (Fig. 3a), with the exception of a drop on day -6. In association with the onset of the preovulatory LH surge on day -2, inhibin A levels on day -3 (132.9 ± 18.3 pg/ml) dropped significantly ($P<0.05$) to the much lower value of 46.7 ± 9.6 pg/ml by day -1. On days 3 and 5, inhibin A levels were significantly greater ($P<0.05$) than those on day 1 (the lowest value; 46.2 ± 8.8 pg/ml); thereafter, they did not change significantly. Plasma concentrations of total inhibin were generally double the inhibin A concentrations (Fig. 3a), to which they were positively related throughout the estrous cycle ($r=0.46$, $P<0.01$); there were two significant rises in total inhibin level: between days -7 and -2 (excluding days -6 and -5) compared with the value on day -10, and on days 3 and 4 compared with the lowest value on day 0. Inhibin B levels in the circulation were below the detection limit of the assay.

Plasma concentrations of E_2 , progesterone, FSH, and LH

Concentrations of E_2 were significantly greater ($P<0.05$) on day -5 (7.9 ± 1.1 pg/ml) than on days -10 and -7, and they continued to increase until day -3. By day -1,

Figure 3 Plasma concentrations of (a) inhibin A (closed circles) and total inhibin (open circles), (b) estradiol (closed circles) and progesterone (open circles), and (c) FSH (closed circles) and LH (open circles) during the estrous cycle of pigs. Values are means $\pm$ s.e.m. ($n=8$). Hormonal data are aligned relative to detection of ovulation on the basis of 6 h interval scanning (ovulation=hour 0).

E₂ levels dropped significantly ($P < 0.05$) to 1 pg/ml from the day -2 value (20.8 ± 2.6 pg/ml). There was no significant increase in plasma E₂ after ovulation. Plasma progesterone levels began to decrease from day -7 compared with the values on day -10 and were at their minimum between days -5 and -1. After ovulation, they began to rise from day 0 and plateaued on day 7.

Concentrations of FSH in the plasma were ~ 1.5 ng/ml between days -10 and -8. FSH levels then decreased significantly ($P < 0.05$) between days -6 and -3 compared with the value on day -8 (Fig. 3c), but they showed a small but significant increase after detection of estrus (day -2). In the 2 days after ovulation (days 0 and 1), a high FSH concentration (peak value 2.3 ng/ml) was noted, but the value had declined significantly ($P < 0.05$) by day 3. The FSH level then showed no significant changes. The LH concentration showed a clear ($P < 0.05$) peak on day -1, but there were no significant changes during the rest of the cycle. An inverse relationship was found between the levels of FSH and inhibin from days -10 to -3 (inhibin A, $r = -0.47$, $P < 0.01$; total inhibin, $r = -0.38$, $P < 0.01$) and from days 0 to 9 (inhibin A, $r = -0.42$, $P < 0.01$; total inhibin, $r = -0.38$, $P < 0.01$). There was also an inverse relationship between FSH and E₂ levels between days -10 and -3 ($r = -0.38$, $P < 0.01$); however, there was no significant correlation between the two hormones from days 0 to 9 ($r = -0.12$, $P \geq 0.1$).

Discussion

Our most important findings were that 1) in terms of specificity and sensitivity, our Tr-IFMA was applicable to measurement of porcine inhibin A in the circulation; and 2) circulating levels of inhibin A were inversely correlated with FSH levels during the follicular growth that occurred between the late luteal and follicular phases of the porcine estrous cycle and during the early luteal phase.

IFMA and FIA recognized 30, 50, 72, and 108 kDa molecules in the eluates obtained from pFF after immunoaffinity chromatography. These are likely to correspond to the higher molecular weight precursor forms of inhibin A, in addition to fully processed 32 kDa inhibin A, as previously described in porcine (Miyamoto *et al.* 1986) and bovine follicular fluid (FF; Sugino *et al.* 1992). Early studies have demonstrated the existence of high concentrations of a 26 kDa-free α subunit (pro- α C) in bovine FF (Knight *et al.* 1989, Robertson *et al.* 1989, Sugino *et al.* 1989) and in the circulation (Knight *et al.* 1989). However, inhibin A IFMA detected little immunoreactivity around the 26 kDa fractions obtained from pFF. The results suggested that 26 kDa pro- α C does not interfere with porcine inhibin A IFMA. Measurement of dimeric inhibins in the fractions of pFF also clearly indicated that porcine follicles produced largely

inhibin A, although smaller amounts of inhibin B were synthesized. These results are consistent with the previous finding that inhibin β_A mRNA expression in porcine follicles is higher than that of the β_B subunit (Li *et al.* 1997). As in cows (Bleach *et al.* 2001, Kaneko *et al.* 2002a, Kaneko & Hasegawa 2007), ewes (Knight *et al.* 1998), and female goats (Medan *et al.* 2003), in sows the inhibin isoform detectable in the circulation is inhibin A; this is in contrast to the case in women (Groome *et al.* 1996,) and female rats (Woodruff *et al.* 1996), in which both inhibin A and B circulate in the peripheral blood.

To our knowledge, this is the first report to describe the correlation between circulating inhibin A and plasma FSH levels and follicular dynamics throughout the pig estrous cycle. An increase in the concentration of plasma inhibin A occurred at the same time as the increase in the number of small follicles between the late luteal and follicular phases and during the early luteal phase. A clear inverse relationship between plasma inhibin A and FSH existed in these two periods, whereas E₂ levels showed no increase and no relationship with FSH concentrations during the early luteal phase. The results are consistent with the detection of relatively constant levels of mRNAs encoding inhibin α and β_A subunits in follicles 1 mm or more in diameter (Guthrie *et al.* 1992, Garrett *et al.* 2000); expression of aromatase mRNA dramatically increases once the follicles reach 6 mm in diameter (Liu *et al.* 2000). Consideration of these findings in combination with the finding that treatment of pigs with inhibin preparations decreases FSH secretion (Guthrie *et al.* 1987, 1988, Knox *et al.* 1991, Knox & Zimmerman 1993, Bracken *et al.* 2006) strongly suggests that inhibin A acts as a feedback regulator of FSH throughout the porcine estrous cycle; in contrast, E₂ has inhibitory effects only during the follicular phase.

The CL of the human and other primates produces large amounts of inhibin-free α subunits (Knight *et al.* 1992) in addition to inhibin A (Schwall *et al.* 1990, Roberts *et al.* 1993), and high concentrations of total inhibin and inhibin A have been noted during the mid-luteal phase of the menstrual cycle (Groome *et al.* 1994, 1996). We found no parallel changes between the plasma concentrations of total inhibin and inhibin A and those of progesterone. The porcine CL does not seem to secrete substantial amounts of inhibins into the circulation.

In our ultrasonographic examinations, mean number of small follicles (≥ 3 and < 6 mm in diameter) detected during the late luteal phase ranged from 20 to 26, which is less than the number (40–50) of the same-sized follicles previously determined by macroscopic measurements (Clark *et al.* 1975, Guthrie *et al.* 1995b). One possible reason for this difference is the fact that our ultrasonographic estimates of follicle size were about 0.7 mm smaller than the macroscopic measurements,

probably because only the antral cavities (i.e. the anechoic regions) of the follicles were measured by the ultrasound scanner. When we rearranged our ultrasonographic data collected during the late luteal phase, the number of follicles ≥ 2 and < 5 mm in diameter, which corresponds approximately to follicles ≥ 3 and < 6 mm in actual diameter, ranged from 32.4 ± 4.9 ($n=8$) to 35.8 ± 3.8 ($n=8$). These follicle numbers are 75–80% of those reported in the previous studies (Clark *et al.* 1975, Guthrie *et al.* 1995b); they are similar to those given in a recent study in which $\sim 80\%$ of follicles between 2 and 4 mm in diameter were detectable by ultrasonography (Gonzalez-Añover *et al.* 2009). Differences in breeds and nutritional conditions between our study and these previous ones may also have affected the number of follicles in the ovary.

Although with ultrasonography not all of the follicles present in the ovary are detectable, our follicle counts, in combination with the changes in plasma inhibin and FSH levels, demonstrated that a group of follicles grew during the early luteal phase and in the late luteal to follicular phases. Follicular growth before ovulation was wave like; it was characterized by the appearance of a cohort of follicles of 3–6 mm in diameter, of which at least 10–17 continued to grow as co-dominant follicles, reaching ovulation while the others regressed, as described in previous reports (Clark *et al.* 1975, Kelly *et al.* 1988a, Guthrie & Bolt 1990, Ryan *et al.* 1994). The high concentrations of FSH that we observed in the mid-luteal phase are likely to stimulate follicle emergence from the late luteal to the follicular phases. These follicles are likely to lower the plasma FSH level by increasing their secretion of inhibin A and, subsequently, E_2 ; thereby, they initiate follicle selection within their own group and suppress the growth of new follicles.

However, there have been controversial findings with regard to the presence of synchronous growth of follicles during the early luteal phase in pigs. Several studies based on counts of the number of follicles in the ovary (Clark *et al.* 1975, Kelly *et al.* 1988a) or ultrasound observations of the ovary (Ryan *et al.* 1994) have suggested that there is no wave-like follicular growth during the luteal phase, apart from the ovulatory follicular wave that appears in the late luteal phase. In contrast, Dalin (1987) showed a trend toward a follicular wave between days 6 and 8 after estrus. We found that the mean concentrations of inhibin A and total inhibin were clearly elevated in the early luteal phase, concomitant with an increase in the number of follicles 3–5 mm in diameter. Combined with the previous findings that mRNAs encoding inhibin α and β_A subunits are expressed in follicles with diameters of 1 mm or more (Guthrie *et al.* 1992, Garrett *et al.* 2000), our findings support the presence of synchronous growth of follicles during the early luteal phase. The fate of these new follicles is not clear from the mean inhibin profiles, because these profiles showed no

significant changes following the transient rise on days 3 and 4. However, analysis of inhibin profiles during the early luteal phase in individual sows indicated that inhibin A and total inhibin showed an elevation around days 3–6 but declined by day 7 as shown in [Supplementary Figure 1](#). Biochemical classification of follicle groups has revealed that most follicles 3–5 mm in diameter are nonatretic on day 5 after estrus in pigs, but that the incidence of follicular atresia increases by day 7 (Guthrie *et al.* 1995a, 1995b). Our inhibin data obtained from each sow, together with the data from these previous biochemical analyses of follicles, suggest that new, small follicles seem to become atretic without further growth, probably because of lowered LH pulse frequency (Flowers *et al.* 1991, Estienne *et al.* 1998, Ferguson *et al.* 2003) under high progesterone levels, because high LH pulse frequency is necessary for continued growth of follicles (Esbenshade *et al.* 1990, Lucy *et al.* 2001).

The typical follicular wave, for example in cattle, is defined as the synchronous growth of a cohort of follicles, one of which continues to grow to a dominant follicle while the others regress (Evans 2003). During the early luteal phase of the porcine estrous cycle, growth of ovulatory-sized follicles is absent (this study plus those of Clark *et al.* (1975), Kelly *et al.* (1988a) and Ryan *et al.* (1994)). However, our data indicate that the cohort of small follicles, the growth of which is stimulated by the secondary FSH surge, has the ability to produce inhibin A and is likely to exert an influence over less mature follicles by lowering FSH secretion. This is similar to the influence proposed for dominant follicles in ruminants (Ko *et al.* 1991, Adams *et al.* 1992). Follicular growth during the early luteal phase in pigs may be functionally analogous to the wave during the early luteal phase of cows (Bleach *et al.* 2001, Kaneko *et al.* 2002a).

To summarize, combined analyses of plasma inhibin A and follicular dynamics revealed two periods of follicle emergence during the porcine estrous cycle. A reciprocal relationship exists between the patterns of secretion of inhibin A and FSH during each follicle emergence, indicating that inhibin A is a primary feedback hormone for FSH in pigs.

Materials and Methods

Animals

Protocols for the use of animals were approved by the Institutional Care and Use Committee for Laboratory Animals of the National Institute of Animal Health, Tsukuba, Japan. Eight sows (crossbreeds of Landrace $\times$ Large White, purchased from Cimco, Tokyo, Japan), weighing 212.8 ± 7.3 kg (mean $\pm$ S.E.M.) and aged 23.3 ± 1.6 months, were used. Mean length of their estrous cycles was 21.3 ± 0.3 days ($n=11$ cycles).

Ovarian examination and blood samplings

Ovaries of sows were scanned daily transrectally with an ultrasound scanner (SSD-900SE, Aloka, Tokyo, Japan) with a 7.5 MHz linear array transducer, starting 10 days after the previous ovulation and ending 10 days after the subsequent ovulation. During examination, images of ovarian structures were recorded on a digital video recorder (MOVIE VISION, System TALKS, Tokyo, Japan). Follicles were identified as anechoic spherical structures; the diameters of follicles 2 mm or more in diameter, and CLs, were measured. Follicles were classified by size as small (≥ 3 and < 6 mm in diameter) or large (≥ 6 mm in diameter), because E_2 production is much higher in follicles at least 6 mm in diameter than in smaller follicles (van de Wiel *et al.* 1983, Kita *et al.* 1994, Shores & Hunter 1999, Liu *et al.* 2000). Growth and regression of CLs were expressed in terms of the mean diameter of the largest CL in each sow on each day. In addition, after sows had been deemed to be in estrus (as defined in 6-hourly observations by their first standing response to an intact boar), the ovaries were scanned at 6 h intervals to detect ovulation. Ovulation was defined as the time when there was a marked reduction in the number of large follicles relative to that in previous observations (Knox & Probst-Miller 2004).

Blood samples were collected every 12 h throughout the study via an indwelling catheter in the auricular vein, and every 6 h from 17 days after the previous ovulation to the end of the subsequent estrus, to characterize hormonal profiles during the periovulatory period. Plasma was recovered after centrifugation of blood and stored at -20°C .

Validation of ultrasonographic measurements of follicular diameter

Porcine ovaries obtained from a local abattoir were scanned in a water bath, and the diameters of 41 follicles were recorded. These follicles were dissected from the ovaries and their diameters were measured by a metallic calliper. Then we compared the diameters of follicles measured by ultrasonography with the diameters of the same follicles after dissection.

Time-resolved FIA of porcine total inhibin

Concentrations of total inhibin were determined by a competitive immunoassay using europium (Eu)-labeled bovine inhibin A as a probe (Kaneko *et al.* 2002b, Ohnuma *et al.* 2007). In time-resolved FIA (Tr-FIA) of porcine total inhibin, anti-bovine inhibin serum (TNDH-1, Hamada *et al.* 1989), provided by Dr K Taya of Tokyo University of Agriculture and Technology, Fuchu, Tokyo, Japan, was used as a primary antibody. Porcine 30 kDa inhibin A, purified from pFF by the method of Hasegawa *et al.* (1994), was used as a reference standard. The detection limit of the assay was 0.039 ng/ml. When total inhibin was measured in a plasma sample (100 μl), serum from an ovariectomized gilt (100 μl) was added to each well of the standards to correct for the matrix effects of porcine plasma. Fluorescence was measured with a fluorometer (Arvo, PerkinElmer Japan, Yokohama, Kanagawa, Japan). The intra- and inter-assay coefficients of variation (CV) were 10.7 and 15.8%, respectively.

Tr-IFMA of porcine inhibin A

Concentrations of inhibin A were determined by Tr-IFMA using purified polyclonal antibodies to the α and β_A subunits of bovine inhibin A, as described previously (Kaneko *et al.* 2002a). Porcine 30 kDa inhibin A was used as a reference standard. Inhibin A IFMA shows very low cross-reactivity ($< 1\%$) with porcine inhibin B, bovine activin A, bovine activin B, bovine activin AB, and bovine pro- αC when the cross-reactivity with porcine inhibin A is calculated to be 100% (Ohnuma *et al.* 2007). The detection limit of the assay was 10 pg/ml. When inhibin A was measured in plasma samples (100 μl), serum from an ovariectomized gilt (100 μl) was added to each well of the standards to correct for the matrix effects of porcine plasma. Serial dilution of plasma samples showed a dose-response curve that paralleled a standard curve generated with porcine inhibin A. The intra- and inter-assay CV were 9.7 and 15.1%, respectively.

Tr-IFMA of porcine inhibin B

Concentrations of inhibin B were determined by Tr-IFMA, as described previously (Kaneko *et al.* 2003). Porcine 29 kDa inhibin B, purified from pFF by the method of Hasegawa *et al.* (1994), was used as a reference standard. Inhibin B IFMA shows very low cross-reactivity ($< 2\%$) with porcine inhibin A, bovine activin A, bovine activin B, bovine activin AB, and bovine pro- αC (Ohnuma *et al.* 2007). The detection limit of the assay was 0.78 ng/ml. The intra- and inter-assay CV were 11.7 and 16.8%, respectively.

Isolation of inhibin molecules from pFF

To determine whether different molecular weight forms of inhibin cross-react in the FIA or IFMAs, 6 μg inhibin, purified from pFF by immunoaffinity chromatography, was fractionated by SDS-PAGE as described previously (Kaneko *et al.* 2002a, 2003). Antiserum used for immunoaffinity chromatography was raised against bovine 32 kDa inhibin (Kaneko *et al.* 1993) and cross-reacting inhibin α subunits (Kaneko *et al.* 2002a, 2003, Ohnuma *et al.* 2007). The gel was cut into 2.0 mm slices. Under gentle shaking overnight, inhibin was extracted from each gel slice with Tris-buffered saline (0.05 M Tris/HCl (pH 7.5) and 0.15 M NaCl) containing 5 mM EDTA. The gel eluates were assayed for total inhibin by FIA and for inhibin A and B by IFMA.

Tr-FIA of porcine FSH, LH, E_2 , and progesterone

Concentrations of FSH or LH in plasma of sows were determined by competitive immunoassays using Eu-labeled FSH (Kaneko *et al.* 2002a, Ohnuma *et al.* 2007) or LH (Noguchi *et al.* 2007) as probes. In the FIA of porcine FSH, anti-porcine FSH serum (AFP-2062096) was used as a primary antibody and porcine FSH antigen (AFP-10640B) was used for Eu labeling and as a reference standard. In the FIA of porcine LH, anti-porcine LH antibody (AFP15103194Rb) was used as a primary antibody and porcine LH (AFP11043B) was used for Eu labeling.

and as the reference standard. Porcine FSH and LH immunoassay kits were provided by Dr A F Parlow of the National Hormone and Peptide Program, Harbor-UCLA Medical Center, Torrance, CA, USA. The respective intra- and inter-assay CV were 7.2 and 10.0% for FSH and 6.8 and 8.4% for LH.

Plasma concentrations of E₂ and progesterone were measured with a Tr-FIA kit (DELFA Estradiol and Progesterone kits, PerkinElmer Japan), as previously reported (Noguchi *et al.* 2007). E₂ and progesterone were extracted from the plasma samples with diethyl ether before being applied to the kits. The respective intra- and inter-assay CV were 8.4 and 11.0% for E₂ and 7.8 and 7.2% for progesterone.

Data analyses

In order to correlate hormonal profiles with follicular growth, data were aligned relative to the detection of ovulation on the basis of 6 h interval scanning (ovulation=hour 0). The estrous cycle was divided into the two periods: before ovulation (days -10 to 0) and after ovulation (days 0-9). Data pertaining to follicular growth and hormonal profiles in each period were subjected to ANOVA for repeated measures (Glantz & Slinker 1990). When a significant effect was detected by ANOVA, the significance of the difference between means was determined by Tukey's test. Regression analyses among inhibin, E₂, and FSH concentrations were also performed. All data were analyzed by using the General Linear Models or REG Procedure of Statistical Analysis Systems (SAS Inc., Cary, NC, USA). A value of $P < 0.05$ was considered to indicate statistical significance.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-09-0018>.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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