Changes of gonadotrophin surge inhibiting/attenuating factor activity in pig follicular fluid in relation to follicle size

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The activity of GnSI/AF was measured in pig follicular fluid (pFF) from 58 individual follicles of various sizes, by bioassay using rat pituitary cells, to investigate the relationship between gonadotrophin surge inhibiting/attenuating factor (GnSI/AF) activity and follicular development. In addition, the correlation between GnSI/AF and inhibin activities and the content of sex steroids (oestradiol, progesterone and testosterone) of follicles was examined. The activity of GnSI/AF in pFF varied significantly ($0.155\text{--}1.69\text{ U }\mu\text{l}^{-1}$) with size of the follicle. The activities (mean $\pm$ SEM) were intermediate and constant in follicles with diameters from 3 to 5 mm ($0.583 \pm 0.080\text{ U }\mu\text{l}^{-1}$, $n = 24$), were higher and reached the highest value in follicles with diameters between 6 and 8 mm ($0.863 \pm 0.068\text{ U }\mu\text{l}^{-1}$, $n = 21$), and were lower, reaching the lowest value in follicles with diameters of 9 and 10 mm ($0.401 \pm 0.089\text{ U }\mu\text{l}^{-1}$, $n = 13$). In contrast, inhibin activity was almost constant during the development of follicles, although individual values varied from 0.9 to $2.5\text{ U }\mu\text{l}^{-1}$. For follicles with diameters of 4–8 mm, inhibin activity was $1.754 \pm 0.042\text{ U }\mu\text{l}^{-1}$ ($n = 39$); activity was higher in the smallest follicles with diameters of 3 mm ($2.063 \pm 0.015\text{ U }\mu\text{l}^{-1}$, $n = 6$) and was lower in follicles with diameter of 9 mm, reaching the lowest value in follicles with diameter of 10 mm ($1.176 \pm 0.068\text{ U }\mu\text{l}^{-1}$, $n = 7$); inhibin activity was not significantly correlated with GnSI/AF activity. Steroid concentration increased in a similar pattern to that of GnSI/AF activity, but no marked decrease was noticed in the large follicles. These characteristic changes of the activity of GnSI/AF of follicles during development, especially in the preovulatory stage, suggest that GnSI/AF plays a role in the ovulatory process as an ovarian regulator for the timely occurrence of the LH surge.

Introduction

The mid-cycle LH surge is thought to be induced by the positive feedback action of oestrogen on the hypothalamic–pituitary axis (Knobil and Hotchkiss, 1988). However, many workers reported the lack of an LH surge, in spite of high concentrations of serum oestradiol, particularly when stimulated by human menopausal gonadotrophin (hMG) or FSH administration (Schenken and Hodgen, 1983; Ferraretti *et al.*, 1983; Littman and Hodgen, 1984). It has been shown that such phenomena during hMG or FSH administration may be achieved through the action of undefined ovarian non-steroidal factor(s), which reduce(s) the pituitary response to GnRH. This factor is called gonadotrophin surge-inhibiting factor (GnSIF) (Sopelak and Hodgen, 1984; Koppelaar *et al.*, 1992), gonadotrophin surge-attenuating factor (GnSAF) (Messinis and Templeton, 1989; Knight *et al.*, 1990), or gonadotrophin surge inhibiting/attenuating factor (GnSI/AF) (Culler, 1992). GnSI/AF was shown to be present in follicular fluid in pigs (Lagace *et al.*, 1979; Danforth *et al.*, 1987), monkeys (Schenken and Hodgen,

1983) and humans (Knight *et al.*, 1990), and was demonstrated to be produced mainly in the ovaries of rats (de Koning *et al.*, 1987) and monkeys (Littman and Hodgen, 1984). However, the physiological significance of this factor, as well as its structural identification, is still to be clarified.

In this study, the relationship between GnSI/AF activity and follicular development was investigated by evaluating the assay conditions for GnSI/AF bioactivity of fluid from follicles of various sizes and measuring their activity throughout follicular development. In addition, the activity of inhibin and concentrations of sex steroids were monitored.

Materials and Methods

Isolation of pFF

Pig ovaries were obtained from a local abattoir and transported to the laboratory in ice-cold 0.9% saline. Follicles were removed and classified according to their diameters. Fluid was collected from each follicle by aspiration with a needle. After centrifugation at 300 g for 5 min, the supernatant was stored at

– 20°C until assay. Atretic follicles were excluded according to the macroscopic criteria of Moore (1978) and follicles containing oestradiol below the concentration of the mean – two SD of each class of diameter were also excluded. In all, 58 follicles were examined (6 to 11 follicles per size from 16 ovaries). In addition, for the reference preparation of GnSI/AF and inhibin activities, a single batch of pig follicular fluid (pFF) was collected from follicles of various (mainly large) sizes.

Pituitary cell culture

Dispersed anterior pituitary cells were prepared according to the method of Vale *et al.* (1972), with minor modifications. Briefly, anterior pituitaries were removed from adult female Wistar rats (240–270 g), minced into small pieces, and dispersed in two steps. First, pituitary cells were incubated in Dulbecco's modified Eagle's medium (DMEM, Nissui Pharmaceutical Co. Ltd, Tokyo) containing 0.2% collagenase (Sigma Chemical Co., St Louis, MO), 0.2% hyaluronidase (Sigma), 10% fetal calf serum (FCS) (Gibco, Grand Island, NY), 100 U penicillin ml⁻¹ and 100 µg streptomycin ml⁻¹ (Meiji Seika Kaisha Ltd, Tokyo) at 37°C for 30 min, and were then incubated in 10 mmol l⁻¹ Ca²⁺- and Mg²⁺-free PBS(–) containing 0.1% trypsin (Dojindo Laboratories, Kumamoto) and 0.001% DNAase I (Sigma) at 37°C for 10 min with gentle agitation. After dispersion, cells were washed and suspended in the medium (DMEM containing 10% fetal calf serum and penicillin/streptomycin) at a concentration of 5×10^5 cells ml⁻¹. The cell suspension was plated to each well (250 µl per well) of multi-well cell culture clusters (Costar, Cambridge, MA). Viability of dispersed cells was > 95% by trypan blue exclusion test.

Charcoal treatment of pFF

To remove steroids, pFF was treated before bioassay with dextran-coated charcoal (Norit A, Nacalai Tesque Inc., Tokyo, pretreated with 10% dextran (molecular weight 50 000–70 000, Nacalai Tesque)). pFF was mixed with dextran-coated charcoal, rotated overnight at 4°C, and centrifuged at 3000 g for 30 min. Supernatant was filtered through a 0.22 µm filter and stored at – 20°C.

The reference preparation of GnSI/AF and inhibin activities

The pooled pig follicular fluid (pFF) described above was treated with dextran-coated charcoal at 40 mg charcoal ml⁻¹ pFF, and was referred to as the reference pFF. By this treatment, oestradiol, progesterone and testosterone concentrations of this reference pFF were reduced below the sensitivities of the radioimmunoassays.

Bioassay of GnSI/AF and inhibin activities

GnSI/AF and inhibin bioactivities were measured according to the method of Danforth *et al.* (1987), except that each bioassay was performed separately because of the marked difference of the optimum assay range between them.

GnSI/AF activity was measured as suppression of GnRH-induced LH secretion. After plating for 48 h, cells were washed twice with fresh medium and incubated in medium with

samples (at a final concentration of 0.2% (v/v), unless otherwise stated) for 48 h. Cells were then washed twice with the fresh medium and incubated with samples in the presence or absence of GnRH (10 nmol l⁻¹) for 4 h. The medium was collected and stored at – 20°C until assayed for LH. The difference of LH secretion in the presence and absence of GnRH was referred to as GnRH-induced LH secretion. GnRH-induced secretion without samples throughout the incubation was referred to as the control.

Inhibin activity was determined by suppression of basal FSH secretion. After plating for 48 h, cells were washed twice with fresh medium and incubated in the medium with or without (control) samples (at the final concentration of 0.02%, v/v, 10% of that for GnSI/AF bioassay, unless otherwise stated) for 48 h; the medium was then collected and stored at – 20°C until assayed for FSH.

Both bioassays were carried out in triplicate for controls and samples.

Radioimmunoassays of hormones

Assays for rat LH and FSH were performed in duplicate using the LH and FSH radioimmunoassay kit generously provided by National Hormone and Pituitary Program, NIDDK. Rat-LH-RP-3 and Rat-FSH-RP-2 were used as the reference standard. The minimum detectable concentrations of LH and FSH were 10 pmol l⁻¹ and 14 pmol l⁻¹ and the intra- and interassay coefficients of variance were 1.8% and 2.0% for LH and 5.8% and 12.8% for FSH, respectively. Assays for oestradiol, progesterone and testosterone were performed in duplicate using commercial radioimmunoassay kits from Diagnostic Products Corporation (Los Angeles, CA). The intra-assay coefficients of variation were less than 10%. The sensitivities of the assays were 30 pmol l⁻¹, 160 pmol l⁻¹ and 140 pmol l⁻¹, respectively.

Statistical analysis

Mean concentrations of gonadotrophin affected by reference pFF and steroids were analysed by Dunnett's multiple range test. Mean concentrations of LH affected by dextran-coated charcoal treated steroid rich pFF, and GnSI/AF and inhibin activities in individual pFF were analysed by Duncan's multiple range test. Correlation between GnSI/AF and inhibin activities were analysed by Pearson's correlation coefficient. In all tests, $P < 0.05$ was considered significant.

Calculation of the concentrations and ED₅₀ values of GnSI/AF and inhibin was performed by a linear regression analysis after a log–logit conversion.

Results

Effects of steroids on bioassay

The effect of steroids on the bioassay system was examined. Oestradiol, progesterone and testosterone at various concentrations (10 pmol l⁻¹–10 nmol l⁻¹) were added singly into the incubation medium and gonadotrophin concentrations were measured (Fig. 1a). Although oestradiol and testosterone (≤ 100 pmol l⁻¹), and progesterone (≤ 1 nmol l⁻¹) had no significant effect on GnRH-induced LH secretion,

Fig. 1. The effect of (a) single steroid at concentrations of (▨) 10⁻¹¹, (▤) 10⁻¹⁰, (▥) 10⁻⁹ and (■) 10⁻⁸ mol l⁻¹ in the incubation medium without reference pFF and (b) combination of three steroids at the concentration of (▨) 10⁻¹¹, (▤) 10⁻¹⁰, (▥) 10⁻⁹ and (■) 10⁻⁸ mol l⁻¹ with reference pFF of various concentrations on GnRH-induced LH secretion by rat cultured anterior pituitary cells. The control was the GnRH-induced LH secretion without pFF and steroids. GnRH-induced LH secretions are expressed as the percentage of the mean of the control (mean + SEM, *n* = 3). *Significantly different from the control value (Dunnett's multiple range test, **P* < 0.05, ***P* < 0.01). Ctrl: control; E₂: oestradiol; P: progesterone; T: testosterone.

oestradiol at 1 nmol l⁻¹, progesterone at ≥ 10 nmol l⁻¹ and testosterone at ≥ 1 nmol l⁻¹ reduced it significantly (*P* < 0.05). Oestradiol, progesterone and testosterone in combination at the concentration of 100 pmol l⁻¹ in the incubation medium with reference pFF at various concentrations (0.02–2.0%) had no significant effect on GnRH-induced LH secretion, whereas > 1 nmol l⁻¹ had a significant suppressive effect (*P* < 0.05) (Fig. 1b).

However, oestradiol, progesterone and testosterone at high concentrations (≥ 10 nmol l⁻¹) had stimulated basal FSH secretion, but these steroids added either singly or in combination at low concentration (10 pmol l⁻¹, which was equivalent to that for GnSI/AF bioassay because sample concentration in incubation medium for inhibin bioassay was 10% of that for GnSI/AF bioassay) had no significant effect on basal FSH secretion (data not shown).

Effect of treatment with dextran-coated charcoal on GnSI/AF activity and sex steroid concentration in pFF

The reference pFF, which was essentially steroid free, was treated again with various concentrations of dextran-coated charcoal (0–1000 mg charcoal ml⁻¹ pFF) and GnSI/AF activity was measured (Fig. 2). While treatment of the reference pFF with dextran-coated charcoal at concentrations ≤ 100 mg ml⁻¹ did not reduce GnSI/AF activity significantly, dextran-

coated charcoal treatment at ≥ 400 mg ml⁻¹ reduced it significantly (*P* < 0.05).

Steroid concentrations of fluid of large size follicles were measured by radioimmunoassay separately and FF containing high concentrations of steroids were chosen and mixed together to make a steroid-rich pFF. This steroid-rich pFF was treated with various concentrations (0–1000 mg ml⁻¹) of dextran-coated charcoal and GnSI/AF activity and oestradiol, progesterone and testosterone concentrations were measured (Fig. 3). Although steroid concentrations of steroid-rich pFF were very high (oestradiol: 759 nmol l⁻¹, progesterone: 1.99 μmol l⁻¹, testosterone: 805 nmol l⁻¹), they were reduced drastically by dextran-coated charcoal treatment with increasing dextran-coated charcoal concentration. Treatment with dextran-coated charcoal at concentrations ≥ 40 mg ml⁻¹ reduced all steroid concentration of pFF to < 30 nmol l⁻¹. This concentration of steroids was equivalent to 60 pmol l⁻¹ in the assay medium because the sample was finally diluted to 0.2% when added into it, and was negligible for our bioassay as demonstrated above. The response curve of GnSI/AF activity to treatment with dextran-coated charcoal consisted of three segments, the first decrease of GnSI/AF activity (1–10 mg dextran-coated charcoal ml⁻¹), the stable segment (10–100 mg ml⁻¹) and the second decrease (> 100 mg ml⁻¹).

From these results, 40 mg ml⁻¹ was chosen as the optimum concentration of dextran-coated charcoal that nullified the effect of steroids and preserved GnSI/AF activity in pFF.

Fig. 2. The effect of treatment with dextran-coated charcoal on gonadotrophin surge inhibiting/attenuating factor (GnSI/AF) activity in the reference pig follicular fluid. Results are expressed as the percentage of the mean of the control (without pFF) (mean $\pm$ SEM, $n=3$). Significantly different from untreated groups (Dunnett's multiple range test, * $P < 0.05$, ** $P < 0.01$).

Dose-response curve of reference pFF on gonadotrophin secretion by cultured pituitary cells and calculation of GnSI/AF and inhibin activities

The reference pFF, which had been treated with the optimum concentration of dextran-coated charcoal at 40 mg ml^{-1} , caused a dose-dependent inhibition of both basal FSH secretion and GnRH-induced LH secretion (Fig. 4). The control study using 2% of heat-treated (at 56°C for 30 min) dextran coated charcoal treated pig serum indicates that protein loading alone was not a contributory factor. This dose-response curve of the reference pFF was referred to as the standard curve for GnSI/AF and inhibin bioassay. After a log-logit conversion, the linear segments of the curve (five points between 0.02 and 2.0% for GnSI/AF, and between 0.005 and 0.02% for inhibin) were analysed for a linear regression. Results of both bioassays are shown (Table 1): 0.2% of the reference pFF in incubation medium caused approximately 50% suppression of GnRH-induced LH secretion (median effective dose; ED_{50}), and 0.01% of reference pFF also caused approximately 50% suppression of basal FSH secretion (ED_{50} of inhibin activity). Because of the difference of ED_{50} values, GnSI/AF and inhibin activities of individual pFF were assayed separately. One microlitre of the reference pFF was defined to contain 1 unit of GnSI/AF activity because 0.2% reference pFF in incubation medium caused 50% suppression of GnRH-induced LH secretion (Danforth *et al.*, 1987). One microlitre of the reference pFF was also defined to contain 1 unit of inhibin activity for the convenience of expression. Both relative potencies of individual samples were calculated on the basis of this definition.

Fig. 3. The effect of treatment with dextran-coated charcoal on (a) gonadotrophin surge inhibiting/attenuating factor (GnSI/AF) activity and (b) steroid concentration in steroid-rich pig follicular fluid. Results are expressed as the percentage of the mean of the control (without pFF) (mean $\pm$ SEM, $n=3$) for (●) GnSI/AF or as the mean concentrations of (○) oestradiol, (△) progesterone and (□) testosterone measured by radioimmunoassay in duplicate. For GnSI/AF activity, values with different letters are significantly different from one another (Duncan's multiple range test, $P < 0.05$).

GnSI/AF and inhibin activities and steroid concentration in individual pFF in relation to follicular diameters

GnSI/AF activity of individual pFF varied significantly according to follicular diameters and fluctuated between 0.155 and $1.69 \text{ U } \mu\text{l}^{-1}$. The activity (mean $\pm$ SEM) was moderate and almost constant in the follicles with diameters from 3 to 5 mm ($0.583 \pm 0.080 \text{ U } \mu\text{l}^{-1}$, $n=24$), increased to the highest value

Fig. 4. The effect of (●) the reference pFF and the heat-treated and (▲) dextran-coated charcoal treated porcine serum on GnRH-induced LH secretion and (b) the effect of the (○) pFF and (△) pig serum on basal FSH secretion by cultured rat anterior pituitary cells. (○) represents the control value of gonadotrophin secretion without pFF or pig serum. Concentrations of pFF and pig serum are expressed as the percentage in the incubation medium. Gonadotrophin secretions are expressed as the percentage of the mean of the control (without pFF) (mean $\pm$ SEM, $n = 3$). Basal FSH secretion (control value, mean $\pm$ SEM, $n = 3$) was $657 \pm 13.6 \text{ pmol l}^{-1}$ in 48 h, and LH secretion with or without GnRH (10 nmol l^{-1}) (mean $\pm$ SEM, $n = 3$) was $1472 \pm 28.3 \text{ pmol l}^{-1}$ in 4 h and $44.8 \pm 1.72 \text{ pmol l}^{-1}$ in 4 h, respectively.

in the large follicles with diameters from 6 to 8 mm ($0.863 \pm 0.068 \text{ U } \mu\text{l}^{-1}$, $n = 21$) with the peak at 7 mm ($0.953 \pm 0.156 \text{ U } \mu\text{l}^{-1}$, $n = 7$), and then decreased rapidly to the lowest value in the large follicles with diameters of 9 and

Fig. 5. Changes of (a) gonadotrophin surge inhibiting/attenuating factor (GnSI/AF) (●) and (▲) inhibin activities, and (b) steroid concentrations of (○) oestradiol, (△) progesterone and (□) testosterone in individual pig follicles according to their diameters. The individual pFF from follicles classified every 1 mm was assayed for GnSI/AF and inhibin activities and steroids. Results are expressed as the mean $\pm$ SEM of GnSI/AF and inhibin activities and the mean concentrations of steroids. The number of follicles assayed is shown under each diameter. Values with different letters of GnSI/AF and inhibin activities are significantly different from one another (Duncan's multiple range test, $P < 0.05$).

Table 1. Bioassay statistics of gonadotrophin surge inhibiting/attenuating factor (GnSI/AF) and inhibin activities of pig follicular fluid

	GnSI/AF	Inhibin
Standard curve		
Analysed range ($\text{U } \mu\text{l}^{-1}$)	$0.1 < x < 10$	$0.5 < x < 2$
Numbers of points analysed	5	5
Regression line	$\ln x = -0.11y + 6.35$	$\ln x = -0.02y + 0.91$
x ; $\text{U } \mu\text{l}^{-1}$		
y ; % of control		
r^2	0.99	0.99
λ	$-0.0197 \sim -0.00102$	$-0.004 \sim -0.000094$
Cultures dose $^{-1}$	3	3
Samples		
Cultures per sample	3	3
Mean intra-assay CV (%)	16.7	7.6

r^2 : coefficient of determination; λ : index of precision (residual variance/slope); CV: coefficient of variation.

10 mm ($0.401 \pm 0.089 \text{ U } \mu\text{l}^{-1}$, $n = 13$). Although inhibin activity of individual pFF also varied from 0.9 to $2.5 \text{ U } \mu\text{l}^{-1}$, its mean value was almost constant without a peak value during the development of the follicle ($1.754 \pm 0.042 \text{ U } \mu\text{l}^{-1}$, $n = 39$, diameters from 4 to 8 mm), except for high values in the smallest follicles with diameters of 3 mm ($2.063 \pm 0.015 \text{ U } \mu\text{l}^{-1}$, $n = 6$) and gradual decrease from diameters of 9 mm to the lowest value at diameters of 10 mm ($1.176 \pm 0.068 \text{ U } \mu\text{l}^{-1}$, $n = 7$).

There was no significant relationship between GnSI/AF and inhibin activities of individual follicles ($y = 1.471 + 0.3478 \times \text{Pearson's correlation index}$; $r = 0.3821$).

Large increases in steroid concentrations were noticed at follicular diameters from 5 to 6 mm. Oestradiol and testosterone concentrations increased further and reached maximum values at follicle diameters of 7–8 mm. Progesterone concentration increased continuously until a diameter of 10 mm was reached.

Discussion

GnSIF was thought to be the factor that blocks oestrogen-induced gonadotrophin surges in superovulated monkeys (Sopelak and Hodgen, 1984) and GnSAF was also described as a similar factor in women (Messinis and Templeton, 1989). Some other groups have also studied similar factors (Knight *et al.*, 1990; Koppelaar *et al.*, 1992). However, its identification or definite physiological roles are still to be clarified. In the present study, to elucidate the relationship between GnSI/AF activity and follicular development, we examined the appropriate assay condition for the quantification of GnSI/AF activity in follicles of various sizes.

As sex steroids are well known to have direct effects on gonadotrophin secretion by cultured pituitary cells (Schally *et al.*, 1973; Tang and Spies, 1975), treatment with dextran-coated charcoal has generally been used to remove steroids from samples, for example FF or serum (Schenken *et al.*, 1984; Knight *et al.*, 1990; Fowler *et al.*, 1990a) to demonstrate the non-steroidal GnSI/AF. But insufficient dextran-coated charcoal

treatment of pFF may result in residual steroid which will have significant effects on the bioassay, and high concentrations could cause significant loss of GnSI/AF activity, like that of inhibin (Tsonis *et al.*, 1983). Careful determination of the optimum condition of treatment is therefore critical, especially in measuring GnSI/AF activity in samples with a wide range of steroid concentration, as in the present study. As reported previously, high concentrations of steroids had significant effects on the bioassay system for GnSI/AF. The detection limit for steroids, added separately or in combination, to our bioassay system for GnSI/AF was 100 pmol l^{-1} . Steroids at this concentration were also undetected in our bioassay system for inhibin (because the final concentration of pFF in assay medium for inhibin bioassay was 10% of that for GnSI/AF). As expected, high concentrations of dextran-coated charcoal ($\geq 400 \text{ mg ml}^{-1}$, charcoal/pFF) of pFF caused significant loss of GnSI/AF activity, and the maximum concentration to preserve GnSI/AF activity was 100 mg ml^{-1} . The minimum concentration of dextran-coated charcoal that could reduce steroid concentration of pFF to undetectable concentrations was 40 mg ml^{-1} and treatment at the conventional concentration (about 5 mg ml^{-1}) was shown to be insufficient for steroid rich pFF from large follicles. The response curve of GnSI/AF activity to treatment with dextran-coated charcoal consisted of three segments. From the result, the first decrease of GnSI/AF activity was probably due to a reduction of steroids, and the second decrease was probably due to reduction of GnSI/AF activity by treatment with high concentration of dextran-coated charcoal. The concentration of dextran-coated charcoal for GnSI/AF bioassay was 40 mg ml^{-1} , where the effect of steroids was removed and GnSI/AF activity was preserved.

In summary, (1) there was an increase of GnSI/AF activity accompanied by an increase of steroid concentration in follicles with diameters from 5 to 8 mm; (2) a subsequent rapid decrease of GnSI/AF activity in follicles of 9–10 mm without the decrease of steroid concentration and (3) there was a difference in the changing patterns of GnSI/AF and inhibin activities in relation to follicular size.

There have been few reports about the change of GnSI/AF activity of FF in relation to follicular stage. In rats, this activity in ovarian extracts did not change during the oestrous cycle, while inhibin-like activity was highest in dioestrus 2 and pro-oestrus (de Koning *et al.*, 1989). Others reported that GnSI/AF was preferentially produced by small follicles in superovulated women and monkeys (Fowler *et al.*, 1990b; Schenken *et al.*, 1984). But it is difficult to compare our results with those of others because of considerable differences in methods used.

It has been suggested that GnSI/AF is the regulator of the LH surge (Whitehead, 1990; Messinis and Templeton, 1991). The dynamic change of GnSI/AF activity of pFF during follicular development, especially during the preovulatory period as shown here, is appropriate to explain this hypothesis. If the plasma change of GnSI/AF activity during follicular phase reflects that of FF reported here, the following could be speculated. The steroid concentrations increase according to the follicular development, and oestradiol at the high concentration increases the response of the anterior pituitary to GnRH and induces this positive feedback. The GnSI/AF activity also increases during follicular maturation, maintains the pituitary in a state of low responsiveness to GnRH to prevent the premature LH surge, and increases the gonadotrophin pool of the pituitary. The rapid decrease of GnSI/AF activity just before ovulation at high concentrations of steroids can result in a maximum response of the pituitary. If the GnSI/AF activity decrease occurs before the initiation of the LH surge, the LH surge is triggered. If the decrease occurs during the LH surge, it promotes or enhances the LH surge to achieve sufficient amplitude and duration for adequate ovulation. However, it has been demonstrated that repeated exogenous GnRH injection provoke a large release of LH, possibly without a change of GnSI/AF activity (Koppenaal *et al.*, 1991). It is very difficult to compare this result with those reported here because of marked differences in experimental conditions. Further investigation will be necessary including detection of the plasma change of GnSI/AF activity throughout the menstrual cycle.

A difference was shown between the changing pattern of GnSI/AF and inhibin activities according to the size of the follicle. Inhibin activity in pFF was reported to decrease when measured by bioassay (Anderson and Hoover, 1982), and increase when measured by radioimmunoassay (Guthrie *et al.*, 1992) during follicular maturation. The results reported here are similar to previous reports but inhibin activity measured in the study reported here may be underestimated because the treatment with dextran-coated charcoal was designed for GnSI/AF bioassay and it may be excessive for inhibin bioassay (Tsonis *et al.*, 1983). However, as the effect of steroids on the inhibin bioassay was nullified by our treatment with dextran-coated charcoal, it appears that the overall changing pattern of inhibin activity in relation to follicular size reflects real changes.

The difference between GnSI/AF and inhibin is a matter of controversy. It has been reported that purified inhibin inhibits GnRH-induced LH secretion (Fukuda *et al.*, 1986) and passive immunoneutralization using anti-inhibin α -subunit serum recovers the preovulatory surge of an FSH-treated rat (Culler, 1992). The contribution of inhibin to GnSI/AF activity of pFF must therefore be considered. However, passive immunoneutralization against inhibin altered the GnRH-induced FSH

secretion, but not that of LH secretion (River *et al.*, 1990). Studies *in vitro* have also provided some evidence that GnSI/AF is different from inhibin (Danforth *et al.*, 1987; Busbridge *et al.*, 1990; Fowler *et al.*, 1990a; Knight *et al.*, 1990), and it has been demonstrated that the suppressive effect of GnRH-induced LH secretion of inhibin in pFF is negligible compared with that of GnSI/AF (Danforth *et al.*, 1987). Although the present study was not designed for the elucidation of the difference between GnSI/AF and inhibin, the discrepancy of the changing patterns of GnSI/AF (suppressive effect on GnRH-induced LH secretion) and inhibin (suppressive effect on basal FSH secretion) bioactivities demonstrated in the study reported here suggests the contribution of factors, including GnSI/AF, other than inhibin. We also obtained a crude GnSI/AF fraction with higher GnSI/AF activity and lower inhibin activity in our preliminary preparation from pFF (unpublished data). The final resolution on this matter awaits a structural identification of GnSI/AF.

In conclusion, we have shown the characteristic change of GnSI/AF activity in pFF in detail according to the follicular development. Its increase parallel to that of steroid concentration toward follicular maturation and the subsequent rapid decrease just before ovulation at high concentrations of steroids suggest that GnSI/AF activity plays a role in the ovulatory process as an ovarian regulator for the timely occurrence of the LH surge.

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