Effects of ovine follicular fluid on plasma LH and FSH secretion in ovariectomized ewes to indicate the site of action of inhibin

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Summary. Ovariectomized ewes were given 2 ml s.c. injections of ovine follicular fluid (oFF) (N = 3) or serum (N = 3) and blood samples were collected each day for 3 days. Follicular fluid caused a significant ($P < 0.005$) reduction in FSH within 1 day, but did not affect mean LH values. Two groups of 3 ewes were treated as above but sampled intensively (each 10 min for 6 h) on Days 1 (before treatment) and 4; mean plasma FSH concentration and plasma LH pulse frequency and amplitude were ascertained. Significant ($P < 0.005$) reduction of FSH concentration was seen in the oFF-treated ewes. A non-specific reduction in LH pulse amplitude, but not pulse frequency, was noted in the control ewes. This experiment was repeated with 2 groups of 4 ewes that were conditioned to the experimental environment and effects on LH secretion were not observed in the controls given serum. Treatment with oFF caused a 70% reduction ($P < 0.005$) in plasma FSH and a small (30%) but significant ($P < 0.005$) reduction in mean LH concentrations. The latter was probably associated with a reduction in LH pulse amplitude in 3/4 animals (N.S.) with no change in LH pulse frequency. Treatment with oFF, as in Exp. 1, caused a 95% reduction in FSH values and significant ($P < 0.01$) reduction (32%) of LH pulse amplitude in ovariectomized ewes that had been subjected to hypothalamo-pituitary disconnection and in which gonadotrophin secretion was reinstated with pulses of 250 ng GnRH every 2 h.

These results suggest that proteins from the sheep follicular fluid, including inhibin, act at the pituitary level to inhibit FSH secretion and may have some effects on LH pulse amplitude.

Introduction

Inhibin has been defined as a factor from the gonads which selectively inhibits the secretion of follicle stimulating hormone (FSH) from the pituitary gland (de Jong & Robertson, 1985). That inhibin has a pituitary site of action is amply demonstrated by its action on pituitary cell cultures *in vitro* (Steinberger & Steinberger, 1976; Baker *et al.*, 1976; Lee *et al.*, 1979; de Jong, Smith & van der Molen, 1979; Scott, Burger & Quigg, 1980). Whether or not inhibin has a hypothalamic action to limit the secretion of gonadotrophin-releasing hormone (GnRH) is less certain. Intra-hypothalamic and intra-ventricular injections of inhibin containing material suppressed plasma FSH concentrations but the mechanism for this effect was not clear (Lumpkin, Negro-Vilar, Franchimont & McCann, 1981; Condon, Leipheimer & Curry, 1983). De Greef, de Jong, de Koning, Steenbergen & van der Vaart (1983) sampled hypophysial portal blood in urethane-anaesthetized rats after a single treatment with a partly purified preparation of inhibin and found no effect on GnRH secretion within 6 h. The possibility that longer term inhibin treatment could affect GnRH secretion has

not been examined. High doses of inhibin are known to inhibit both FSH and LH release induced by GnRH injection into ovariectomized ewes (Findlay, Gill & Doughton, 1985). Since LH pulses reflect GnRH secretion (Clarke & Cummins, 1982), it is possible to assess, indirectly, the effects of high doses of inhibin on hypothalamic function by examining LH pulse frequency. McNeilly (1984) showed that follicular fluid from cows did not affect LH pulse amplitude or frequency during the early follicular phase of the sheep oestrous cycle. The interpretation of this study could be confounded by the effects of oestrogen which may increase GnRH pulse frequency at this time (Karsch, Foster, Bittman & Goodman, 1983). Wallace & McNeilly (1985) found that bovine follicular fluid increased LH pulse frequency during the luteal phase of the oestrous cycle of the ewe, an effect that could have been obtained indirectly by a reduction in oestrogen secretion and thus a decrease in oestrogen negative feedback.

We have sought clarification of the site of action of inhibin *in vivo* by measuring LH pulse frequency and amplitude before and after treatment of ovariectomized ewes with ovine follicular fluid (oFF). The pituitary effect of inhibin was defined in ovariectomized ewes that were subjected to hypothalamo-pituitary disconnection (Clarke, Cummins & de Kretser, 1983) and given exogenous GnRH pulses to maintain gonadotrophin secretion. A preliminary account of this work has been presented (Clarke, Findlay & Cummins, 1984b).

Materials and Methods

Animals. Mature Merino ewes were ovariectomized at least 1 month before experimentation by ventral midline incision under pentobarbitone-induced and halothane-maintained anaesthesia. The hypothalamic-pituitary disconnection procedure was performed under halothane anaesthesia, as previously described (Clarke *et al.*, 1983). During experimentation the sheep were housed in individual pens and fed chaffed lucerne with free access to water. The studies were performed in late anoestrus (December).

Injection material. The source of inhibin was fluid aspirated from follicles of sheep ovaries collected at a local abattoir. Materials from two separate collections were used. The follicular fluid was charcoal-stripped and centrifuged according to the method of Tsonis *et al.* (1983) and then subjected to bioassay for inhibin potency (Scott *et al.*, 1980; Lee, Quigg, McMaster, Burger & Leversha, 1983) and oestrogen radioimmunoassay (Carson, Trounson & Findlay, 1982). The inhibin potency of pool 1 (oFF-1) was 21 100 U/ml (15 700–28 500, 95% confidence intervals) and the potency of pool 2 (oFF-2) was 31 500 U/ml (22 700–38 800, 95% confidence intervals) (index of bioassay precision, $\lambda = 0.10$). Both preparations of oFF had an oestradiol-17 β concentration of 0.2 nmol/l. The stripped oFF was stored at -15°C until used. Control animals were treated with serum from hypophysectomized ewes and had no detectable levels of inhibin (<0.004 kU/ml) or oestradiol-17 β (<0.05 pmol/l).

Experiment 1. This experiment was done to verify the result obtained by Findlay *et al.* (1985) especially with respect to the effect of oFF on LH concentrations. Groups of 3 ovariectomized ewes were given 2 ml oFF-1 or 2 ml control serum by s.c. injection beginning at 17:00 h on Day 1 and subsequently at 08:30 and 17:00 h on Days 2 and 3 and at 08:30 h on Day 4. Blood samples were taken by jugular puncture each hour for 6 h between 10:00 and 15:00 h on Days 1, 2, 3 and 4. The plasma was harvested and stored at -15°C for LH and FSH radioimmunoassay.

Experiment 2. The aim of this experiment was to examine the effects of oFF on plasma LH pulse frequency and amplitude, the hypothesis being that alterations in LH pulse frequency would be suggestive of alterations in GnRH pulse frequency. The experiment was done twice because there was a significant change in LH pulse amplitude with time in the serum-treated controls in Exp. 2a.

In Exp. 2a, two groups of 3 ovariectomized ewes were treated with oFF-1 or control serum as in Exp. 1. The sheep were brought in from pasture 3 days before the start of the experiment. Jugular venous catheters (Dwellcath; Tuta Labs Australia Pty Ltd, Sydney, Australia) were introduced and connected to 100 cm manometer lines (Portex Ltd, Hythe, Kent, U.K.). The manometer lines were closed with a 3-way tap and fixed to the sides of the animals' cages; blood samples could therefore be taken without disturbing the sheep. Jugular blood samples (6 ml) were collected at 10-min intervals between 09:00 and 15:00 h on Days 1 and 4. Plasma was harvested and stored at -15°C until assayed for LH and FSH.

In Exp. 2b, sheep were housed and handled for 2 months before being used. These ovariectomized ewes were given s.c. injections of 2 ml oFF-2 ($N = 4$) or control serum ($N = 4$) at 17:00 h on Day 1, at 08:30 and 17:00 h on Day 2 and at 08:30 h on Day 3. The sheep were treated for 1 day less than those in Exp. 2a because of a shortage of ovine follicular fluid and the previous demonstration that oFF had a major effect on FSH by this time. Blood samples were collected (as above) on Days 1 and 3 and the plasma stored at -15°C for LH and FSH assay.

Experiment 3. The aim of this experiment was to determine whether inhibin might act at the pituitary level to reduce LH responses to GnRH *in vivo*. Seven ovariectomized ewes that had also had a hypothalamo-pituitary disconnection received bilateral jugular venous catheters (Silastic tubing, Cat. No. 602-155; Dow Corning Corporation, Midland, MI, U.S.A.). One line was used for blood sampling and the other was connected by manometer line to an infusion pump. The infusion system which has been described previously (Clarke, Cummins, Findlay, Burman & Doughton, 1984a) delivered 250 ng pulses of GnRH in 2.25 ml over 6 min every 2 h. Infusions began 2 days after surgery for disconnection and the experimental period began after 11 days of GnRH replacement. Three sheep received oFF-1 injections s.c. at 17:00 h on Day 1 at 08:30 and 17:00 h on Days 2 and 3 and 08:30 h on Day 4, and 4 sheep received 2 ml injections of control serum at the same times. Blood samples were collected each day between 09:55 and 15:00 h. Samples were taken 5 min before and 15, 30 and 60 min after 3 consecutive GnRH pulses. Plasma was harvested and stored at -15°C for LH and FSH assay.

Radioimmunoassays. Data for hormone assays were computed by the method of Burger, Lee & Rennie (1972).

Plasma LH concentrations were obtained by the method of Lee *et al.* (1976) using NIH-LH-S18 as standard. All samples from a given sheep were assayed in the same run, and were read on that portion of the standard curve where the within-assay coefficient of variation (CV) was $<20\%$. The minimum within-assay CV was 3.8–6.4% between 7.4 and 11.6 ng/ml in 11 assays. Between-assay CV was 10% at 42 ng/ml and 7% at 6.6 ng/ml. Assay sensitivity ranged from 0.2 to 0.6 ng/ml.

Plasma FSH concentrations were obtained by the method of Bremner, Findlay, Lee, de Kretser & Cumming (1980) using NIAMDD-FSH-S13 (biological potency $15 \times$ NIH-FSH-S1) as standard. Samples were assayed and read as for LH (see above). The minimum within-assay CV was 2.6–6.0% between 42 and 53 ng/ml and assay sensitivity was 1.1–1.8 ng/ml ($N = 10$). Between-assay CV was 5% at 64 ng/ml and 7% at 27 ng/ml.

Analysis of data. In ovariectomized ewes LH pulses were defined as previously (Clarke & Cummins, 1985). To calculate LH pulse amplitude we have taken the difference between the maximum value during a pulse and the value immediately before the start of a pulse. Inter-pulse interval was calculated as the time between successive peaks.

Statistics. It was desirable to compare changes in plasma hormone concentrations between two groups of animals when measurements were made before and during treatment and when more than one estimate was made in each animal on each day. To determine whether or not a decrease (one-sided test) with time was greater in treated animals than in controls, we used Student's *t* statistic as follows:

Let A = sum of Day 1 averages

B = sum of Day 4 averages

$C = B - A$

$D = \sum x^2$ for Days 1 and 4, x being a typical reading

$E = N[\sum a^2]$ where N = number of values for each animal on each day and a is the average reading on any day

A^i to E^i are corresponding values for treated animals

$G = ((D + D^i) - (E - E^i))/2(X + Y)(N - 1)$ where X is the number of controls and Y is the number of treated animals.

Then $t = (C - C^i)/\{2G(X + Y)/N\}$ with $2(X + Y)(N - 1)$ degrees of freedom.

Results

Experiment 1: effect of oFF on plasma FSH and LH concentrations

Injection of 2 ml oFF-1 caused a significant ($P < 0.005$) reduction in plasma mean FSH concentrations in ovariectomized ewes within 1 day (Table 1). Concentrations continued to decline

Table 1. Effects of oFF and control on plasma LH and FSH concentrations (means $\pm$ s.e.m.) in ovariectomized sheep (Exp. 1)

Day of exp.	Plasma LH conc. (ng/ml)		Plasma FSH conc. (ng/ml)	
	Control (N = 3)	oFF-1 (N = 3)	Control (N = 3)	oFF-1 (N = 3)
1 (pretreatment)	5.5 $\pm$ 0.8	8.9 $\pm$ 2.2	46 $\pm$ 7	85 $\pm$ 7
2	7.4 $\pm$ 0.6	7.9 $\pm$ 0.9	51 $\pm$ 9	36 $\pm$ 3***
3	8.0 $\pm$ 1.1	7.1 $\pm$ 0.8	52 $\pm$ 9	18 $\pm$ 2***
4	7.5 $\pm$ 2.5	5.9 $\pm$ 0.3	51 $\pm$ 10	12 $\pm$ 2***

*** Significant ($P < 0.005$) fall relative to Day 1 values and relative to the change in control values at the same time.

Table 2. Effects of oFF and control serum on mean ($\pm$ s.e.m.) plasma LH concentrations, LH pulse amplitude, LH inter-pulse interval and plasma FSH concentrations in ovariectomized ewes (Exp. 2)

	No. of ewes	Exp. 2a		No. of ewes	Exp. 2b	
		Day 1	Day 4		Day 1	Day 3
Control serum-treated ewes						
Mean LH (ng/ml)	3	8.7 $\pm$ 1.5	6.9 $\pm$ 0.8*	4	7.5 $\pm$ 1.7	8.6 $\pm$ 2.0
LH pulse						
Amplitude (ng/ml)	3	5.2 $\pm$ 1.2	2.6 $\pm$ 0.6*	4	6.5 $\pm$ 1.0	6.0 $\pm$ 1.4
Interval (min)	3	41 $\pm$ 2	38 $\pm$ 4	4	54 $\pm$ 5	47 $\pm$ 3
Mean FSH (ng/ml)	3	56 $\pm$ 16	58 $\pm$ 15	4	100 $\pm$ 15	99 $\pm$ 16
oFF-treated ewes						
Mean LH (ng/ml)	3	5.0 $\pm$ 1.8	2.6 $\pm$ 0.7	4	5.0 $\pm$ 0.3	3.5 $\pm$ 0.6†
LH pulse						
Amplitude (ng/ml)	3	4.2 $\pm$ 3.0	1.2 $\pm$ 0.2	4	4.6 $\pm$ 0.7	3.6 $\pm$ 1.5
Interval (min)	3	38 $\pm$ 3.0	46 $\pm$ 6	4	51 $\pm$ 4	55 $\pm$ 8
Mean FSH (ng/ml)	3	43 $\pm$ 2.0	—‡	4	64 $\pm$ 10	19 $\pm$ 9†

* Significantly ($P < 0.005$) lower than Day 1 values.

† Significant ($P < 0.005$) fall compared with controls at same time.

‡ Not detectable.

throughout the experiment and by Day 4 the values were 14% of those on Day 1. Mean plasma LH concentrations did not change significantly in these animals and neither did plasma LH nor FSH concentrations fall in the control ewes.

Experiment 2: effects of oFF on plasma LH concentrations

Experiment 2a. After 3 days of oFF treatment, FSH was non-detectable in the plasma of the ovariectomized ewes; values did not change in control ewes (Table 2). There was a significant ($P < 0.005$) reduction in the mean plasma LH concentrations and the LH pulse amplitudes after 4 days of treatment with control serum (Fig. 1), indicating a non-specific effect. Mean plasma LH and LH pulse amplitudes in oFF-treated ewes fell by a similar amount (Table 2). The interval between LH pulses was not affected by treatment with oFF or control serum.

Fig. 1. Plasma LH concentrations before (Day 1) and after (Day 4) control serum treatment of an ovariectomized ewe from Exp. 2a: ▼ indicates a significant pulse as defined in 'Materials and Methods'.

Experiment 2b. Treatment with control serum had no significant effect on the values measured. Treatment with oFF caused a significant ($P < 0.005$) reduction in plasma FSH concentrations (Fig. 2). Also, relative to the change in control animals, oFF treatment caused a significant ($P < 0.005$) reduction in mean LH concentrations. There was no significant effect of oFF on plasma LH pulse amplitude; this result was weighted by 1 ewe in which the amplitude rose from 4.6 ± 0.9 (s.e.m.; 7 pulses) to 8.1 ± 0.8 (5 pulses) ng/ml whereas reductions were seen in 3 other animals. Mean $\pm$ s.e.m. values for these 3 animals were 4.6 ± 0.9 ng/ml on Day 1 and 2.1 ± 0.3 ng/ml on Day 4 ($P < 0.005$ compared to the change in control serum-treated ewes). Intervals between pulses were unaffected by oFF treatment.

Fig. 2. Plasma LH and FSH concentrations in individual ovariectomized ewes before (Day 1) or after (Day 3) treatment with (a) oFF or (b) control serum in Exp. 2b: ▼ indicates a significant LH pulse as defined in 'Materials and Methods'. Note partial scale for FSH values in (b).

Table 3. Effects of oFF and control serum on mean $\pm$ s.e.m. plasma FSH concentrations and plasma LH pulse amplitudes in ovariectomized ewes given 250 ng pulses of GnRH every 2 h

Day of exp.	Control serum-treated ewes (N = 4)		oFF-treated ewes (N = 3)	
	LH pulse amplitude (ng/ml)	FSH (ng/ml)	LH pulse amplitude (ng/ml)	FSH (ng/ml)
1 (no treatment)	7.5 $\pm$ 1.3	58 $\pm$ 3	7.8 $\pm$ 1.0	43 $\pm$ 6
2	8.0 $\pm$ 1.1	59 $\pm$ 2	6.6 $\pm$ 1.0	10 $\pm$ 1***
3	8.5 $\pm$ 1.6	60 $\pm$ 3	5.4 $\pm$ 1.3**	3 $\pm$ 1***
4	7.1 $\pm$ 1.5	63 $\pm$ 2	5.3 $\pm$ 0.7*	2 $\pm$ 1***

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, compared with value for controls at the same time.

Fig. 3. Plasma LH and FSH concentrations in ovariectomized ewes with a hypothalamo-pituitary disconnection given 250 ng pulses of GnRH every 2 h, showing the effects of (a) oFF or (b) control serum in individual animals from Exp. 3. Note partial scale for FSH values in (b).

Experiment 3: direct pituitary effects of oFF

Treatment with control serum did not cause a significant alteration in plasma FSH concentrations and there was no significant change in plasma LH pulse amplitude (Fig. 3). Treatment with oFF caused a significant ($P < 0.005$) reduction in FSH values within 1 day and a significant ($P < 0.01$) reduction in plasma LH pulse amplitude within 2 days. While FSH values fell by 95% over 2 days, the LH values fell by 32%.

Discussion

These studies show that the major effect of inhibin is to reduce FSH secretion by action on the pituitary gland. Since oFF treatment did not affect LH pulse frequency, it may be concluded that inhibin does not act on the hypothalamic or extrahypothalamic centres that determine GnRH pulse frequency. The weight of evidence presented in the present paper suggests that components of oFF can affect LH pulse amplitude, probably by a direct pituitary action.

The in-vivo data presented herein verify that ovine follicular fluid is a potent source of inhibin (Cummins, O'Shea, Bindon, Lee & Findlay, 1983; Findlay *et al.*, 1985). Similar results have been obtained using follicular fluid in rats (de Jong & Sharpe, 1976; Marder, Channing & Schwartz, 1977; Hermans, van Leeuwen, Debets & de Jong, 1980; Hermans, Debets, van Leeuwen & de Jong, 1981; de Greef *et al.*, 1983). Our experiments show that FSH secretion can be virtually stopped by ovine follicular fluid in GnRH-pulsed ovariectomized ewes with a hypothalamo-pituitary disconnection, indicating that a hypothalamic action is not obligatory for inhibin action. Our data do not support the previous findings of Lumpkin *et al.* (1981) and Condon *et al.* (1983) indicating that inhibin has a selective effect on FSH secretion via a hypothalamic mechanism. It does remain possible that inhibin could affect the release of a separate FSH releasing factor that has been postulated (Chappel & Barraclough, 1976) but not identified.

Various studies have shown that follicular fluid preparations may reduce plasma LH concentrations during the periovulatory period in rats (DePaolo, Schander, Wise, Barraclough & Channing, 1979; Rush *et al.*, 1981) or male rats after castration (de Jong & Sharpe, 1976). Others have reported enhancement of LH secretion after follicular fluid treatment (Bronson & Channing, 1978; Miller, Wesson & Ginther, 1979; Wallace & McNeilly, 1985) and some have reported no change (Hoffman, Lorenzen, Weil & Schwartz, 1979; Campbell & Schwartz, 1979; de Greef *et al.*, 1983). In ovariectomized ewes Cummins *et al.* (1983) found a slight suppression of plasma LH after 3 days of oFF treatment. Findlay *et al.* (1985) reported that average LH concentrations could be reduced by high doses of oFF but the statistical methods used did not indicate significant trends. For the present study we have used a more rigorous statistical analysis and a more frequent sampling schedule and found that oFF can cause a significant reduction in LH secretion. This effect was noticed in Exp. 2b and was probably due to a reduction in LH pulse amplitude, which occurred in 3 of 4 animals. The variable patterns of the LH responses to oFF (1 sheep showed an increased amplitude) indicate the fickle nature of the LH secretory mechanisms with regard to follicular fluid proteins. Similarly inconsistent results have been achieved in rats by Charlesworth *et al.* (1984), who explained their results in terms of varying backgrounds of GnRH stimulation.

With particular reference to the sheep, the present results and those of Cummins *et al.* (1983) and Findlay *et al.* (1985), all studies with ovariectomized ewes, differ from those obtained by Wallace & McNeilly (1985), who observed increased LH pulse frequency and amplitude in ewes treated with bovine follicular fluid during the luteal phase of the oestrous cycle. Results with intact ewes are confounded by the effects of ovarian steroids; for example, follicular fluid can reduce plasma FSH concentrations which could in turn reduce oestrogen production from the ovarian follicles (see review by Baird, 1983) leading to a lessening of the negative feedback effect on

GnRH/LH secretion. Since our experiments were not affected by ovarian steroids we were able to ascertain the direct effects of ovine follicular fluid on gonadotrophin secretion.

We found that oFF did not affect LH pulse frequency but did exert a maximal effect on FSH secretion. Therefore, any effect on GnRH output would necessitate a reduction in GnRH pulse amplitude. Although we possess a conscious animal model to measure GnRH secretion we cannot compare the GnRH pulse amplitudes in different sheep (Clarke & Cummins, 1985), and so we do not have a direct way of measuring oFF effects on GnRH pulse amplitude. In the present study we have used an indirect method, that of the sheep with a hypothalamo-pituitary disconnection, and examined the exclusive pituitary action. We account for the reduced LH pulse amplitude seen after oFF treatment of ovariectomized ewes by a pituitary effect of inhibin because a reduction in LH pulse amplitude was seen in GnRH-pulsed, oFF-treated sheep that had been ovariectomized and had a hypothalamo-pituitary disconnection. However, an additional effect on GnRH pulse amplitude cannot be ruled out.

It appears that this effect on LH pulse amplitude is by a mechanism(s) different from those responsible for inhibiting FSH secretion. In general, any hormonal influence that affects both gonadotrophins, e.g. oestrogen, has a more rapid effect on LH than on FSH (Clarke, Funder & Findlay, 1982). In oFF-treated ewes the reverse was noted in Exp. 3 in which an FSH effect was obtained within 1 day but an LH effect was not obtained until 2 days of treatment. This supports the view that inhibin acts selectively on FSH secretory mechanisms, particularly at lower doses of oFF (Findlay *et al.*, 1985).

In the course of one of our experiments (Exp. 2a) we observed a non-specific reduction in the LH pulse amplitude of control ewes treated with serum from an hypophysectomized ewe. These sheep had been recently removed from pasture and placed in individual pens and subjected to a change in diet; we suspect that one or all of these factors were responsible for the observed reduction in LH output. A similar effect was not observed when sheep were conditioned to the experimental environment.

In conclusion we have shown that the action of inhibin to regulate FSH secretion can be accounted for by pituitary action. Inhibin or other follicular fluid proteins do not affect GnRH/LH pulse frequency but may affect LH pulse amplitude. We argue that the latter is probably due to a pituitary effect.

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