Demonstration of a non-steroidal, non-inhibin factor in the ovine corpus luteum of pregnancy that reduces pituitary responsiveness to GnRH-induced LH secretion *in vitro*

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The decline in pulsatile LH secretion and pituitary responsiveness to GnRH as pregnancy advances may be due to non-steroidal factors secreted by the ovine corpus luteum of pregnancy. Corpora lutea from ten ewes on days 70–80 of gestation were homogenized, charcoal-treated and, together with charcoal-treated follicular fluid from super-ovulated women, were subjected to inhibin immunoaffinity chromatography, reducing dimeric inhibin A and B by > 90% and abolishing inhibin bioactivity. These preparations were investigated using cultures of rat pituitary cells. GnRH-induced LH and FSH secretion *in vitro* was reduced by ovine corpus luteum extract and human follicular fluid by $47 \pm 5\%$ and $42 \pm 5\%$ of control LH and by $37 \pm 5\%$ and $50 \pm 10\%$ of control FSH, respectively ($P < 0.001$). Extracts prepared from corpora lutea and placentae that were

collected on days 50, 90 and 120 of pregnancy (five ewes per stage of pregnancy) showed increased GnRH-induced LH-suppressing bioactivity, particularly in the case of the placental extracts, with a threefold increase in activity. When partially purified by pseudochromatofocusing, GnRH-induced LH-suppressing bioactivity in extracts of ovine corpora lutea was identified at pH 5.40 and 5.77. Although these values are similar to published gonadotrophin surge-attenuating factor (GnSAF) bioactivity pI values, a GnSAF-blocking antiserum had no consistent effect on ovine corpus luteum extract GnRH-induced LH-suppressing bioactivity. It was concluded that the ovine corpus luteum of pregnancy contains a non-steroidal, non-inhibin factor, probably not GnSAF, that has the ability to reduce pituitary responsiveness to GnRH *in vitro*.

Introduction

Reduced pituitary responsiveness to GnRH (Chamley *et al.*, 1974; Jenkin *et al.*, 1977) coincides with a reduction in the content of pituitary LH (Chamley *et al.*, 1976; Jenkin *et al.*, 1977) and LH-secreting gonadotroph hypoplasia (Wise *et al.*, 1986) in pregnant sheep. After parturition, GnRH pulse frequency and amplitude increase to cyclic values in ewes (Wise, 1990) and the decreased pituitary LH and mRNA content observed during gestation (Wise *et al.*, 1985) is reversed. Because of the close relationship between GnRH and LH during the ovine oestrous cycle (Levine *et al.*, 1982; Clarke *et al.*, 1987), a reduction in GnRH signalling during gestation may be responsible for lowered LH synthesis. Evidence for this proposal is derived from hypothalamic–pituitary gland disconnected ewes in which the absence of the GnRH signal leads to a reduction in synthesis and secretion of LH (Hamernik *et al.*, 1986). Furthermore, in postpartum ewes, there is an increase in secretion of LH in response to exogenous GnRH (Wright *et al.*, 1981)

and an increase in pituitary LH content (Jenkin *et al.*, 1977), although the number of pituitary GnRH receptors remains similar (Crowder *et al.*, 1982).

Increasing concentrations of steroid hormones during the third trimester of pregnancy in ewes (Thompson and Wagner, 1974; Challis and Patrick, 1981; Thomas *et al.*, 1994) may be responsible for the long-term downregulation of GnRH secretion and the synthesis and release of gonadotrophin. This finding is supported by evidence that progesterone has oestradiol-dependent inhibitory effects on LH secretion and the ability to suppress the ovine oestradiol-induced LH surge (Kasa-Vubu *et al.*, 1992). However, evidence that non-steroidal factors from the ovary may play a role in the inhibition of LH secretion emerged from several investigations in pregnant and postpartum ewes. In a study to elucidate the role of the ovary in the control of LH secretion in the early puerperium in ewes, it was observed that pituitary and serum LH concentrations remain low during the last third of pregnancy. Subsequently, the rate of increase of LH after parturition was lower in intact ewes than in ewes from which the ovaries were removed at day 28 and pregnancy was maintained with progesterone implants

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(O'Reilly and Dziuk, 1973). The concept that non-steroidal factors from the corpus luteum of pregnancy may play a role in the inhibition of pulsatile LH release during pregnancy was proposed by Al-Gubory (1989a). This proposal was based on the observation that the surgical removal of the corpus luteum at mid-pregnancy had no subsequent effects on plasma concentrations of progesterone and FSH, although the frequency of LH pulses increased. Similarly, the frequency of LH pulses increased more rapidly in the puerperium in ewes from which the corpus luteum was removed at mid-pregnancy than in intact ewes (Al-Gubory *et al.*, 1989b). These data were confirmed by a subsequent study demonstrating that charcoal-treated extracts of the corpus luteum of pregnancy, but not the corpus luteum of the oestrous cycle, muscle or fetal cotyledons, inhibited pulsatile LH secretion in ewes (Al-Gubory *et al.*, 1992). In addition, the preliminary observation that serum from women in the third trimester of gestation contains marked gonadotrophin surge-attenuating factor (GnSAF) bioactivity (Fowler *et al.*, 1995; Fowler and Templeton, 1996) introduces the possibility that the corpus luteum of pregnancy or the placenta may be a source of this non-steroidal hormone. Al-Gubory *et al.* (2003) reported that the pulsatile LH release and pituitary responsiveness to GnRH decreases progressively as pregnancy advances, but this study failed to support the hypothesis that ovarian and placental steroids are solely responsible for the inhibition of LH secretion and GnRH-induced LH release during pregnancy in sheep.

The aim of the present study was to determine whether the corpus luteum of pregnancy and placental extracts contain specific bioactivity, distinct from ovarian steroids and inhibin, which could reduce pituitary responsiveness to GnRH *in vitro*.

Materials and Methods

Preparations of ovine corpora lutea and placentae

All procedures relating to the care and use of animals were approved by the French Ministry of Agriculture according to French regulations for animal experimentation. All corpora lutea were collected from ten Prealpes-du-Sud ewes (maintained at INRA, Jouyen-Josas) on days 70–80 of pregnancy. The corpora lutea were surgically removed as described by Al-Gubory *et al.* (1989a), snap-frozen in liquid nitrogen and then stored at -20°C until use. The corpora lutea were thawed and the ovine luteal extracts at days 70–80 of gestation (oLE70–80) were obtained as follows. Corpora lutea were minced and homogenized in 0.9% (w/v) ice-cold saline. The homogenates were stirred for 1 h and centrifuged at 3000 *g* for 30 min. The oLE70–80 preparation was subjected to twofold charcoal treatment (each for 24 h at 4°C with 10 mg charcoal + 1 mg dextran per ml of extract) as described by Fowler *et al.* (1990). Subsequently, ovine

luteal extracts (oLE) and ovine placental extracts (oPE) were prepared from corpora lutea of pregnancy and placental samples collected at days 50, 90 and 120 of gestation (five ewes per stage of pregnancy). The samples were extracted in the same way and protein content determined in ng ml^{-1} (oLE50, 23.3 ng ml^{-1} ; oLE90, 26.4 ng ml^{-1} ; oLE120 19.2 ng ml^{-1} and oPE50, 12.7 ng ml^{-1} ; oPE90, 8.0 ng ml^{-1} ; oPE120, 18.3 ng ml^{-1}). After charcoal treatment, all extracts were centrifuged for 1 h at 105 000 *g* at 4°C before passage through 0.45 μm filters. The resulting extracts were transported to Oxford Brookes University (half the oLE70–80) or the University of Aberdeen (half the oLE70–80 and oLE50, 90, 120 and oPE50, 90, 120) on dry ice.

Human follicular fluid

All protocols using human subjects were given Joint Ethical Committee Approval at Aberdeen and all patients gave informed consent. Over 500 ml human follicular fluid (hFF) was aspirated from follicles of 18 mm in diameter from 40 women undergoing IVF in Aberdeen. The follicles were pooled, treated with charcoal (Fowler *et al.*, 1990) to reduce concentrations of oestradiol and progesterone to $<0.07 \text{ nmol l}^{-1}$ and $<1.3 \text{ nmol l}^{-1}$, respectively, and desalted as described by Fowler *et al.* (2002). An aliquot of 100 ml of this hFF was transported to Oxford Brookes University on dry ice. The hFF was used as a control in this study because it is known to contain a non-inhibin, non-steroidal factor, GnSAF, that reduces GnRH-induced LH secretion (Fowler and Templeton, 1996).

Inhibin depletion of oLE and hFF

Aliquots of 100 ml oLE70–80 and hFF were subjected to inhibin depletion using immobilized R1 monoclonal antibody which recognizes an epitope in the α inhibin subunit (Groome *et al.*, 1995). Each 100 ml aliquot of hFF and oLE was incubated overnight with 2 ml HiTrapTM (Pharmacia, Milton Keynes) NHS-activated gel carrying 20 mg of the R1 antibody, which was then removed by centrifugation (3000 *g*, 30 mins). The process was repeated with fresh regenerated gel and the aliquots of oLE and hFF were then transported to the University of Aberdeen on dry ice. After inhibin depletion, protein concentrations of hFF and oLE were 53 and 12 mg ml^{-1} , respectively. The content of inhibin A and B of the hFF had been reduced by $>90\%$, whereas the inhibin content of the oLE was undetectable after depletion using the assays described by Knight *et al.* (1998).

Rat pituitary cell culture bioassay

Adult female Sprague–Dawley rats were maintained under a constant 12 h light:12 h dark photoperiod at 22°C with access to food and water *ad libitum*. For each

cell culture, 15 rats selected at random during the oestrous cycle were killed by stunning and cervical dislocation. Dispersion and culture of the pituitary cells was carried out as described in Fowler *et al.* (1994) and only preparations with >75% viability of dispersed cells were used for bioassay. Primary pituitary cell cultures were at 100 000 viable cells per 500 μ l culture medium per well in 48-well tissue culture plates. The cells were cultured under sterile conditions for 24 h at 37°C in a water-saturated atmosphere of 5% CO₂ and 95% air mixture with serum-free defined culture medium (SFDM) as defined in Fowler *et al.* (1994). Media and non-attached cells were removed by aspiration and the wells washed with 500 μ l SFDM. All experiments were then carried out on quadruplicate wells as follows: 450 μ l fresh SFDM was added, together with the treatments made up to 50 μ l with SFDM. All the culture plates contained at least four control wells receiving SFDM only. After 24 h of incubation with the test substances, the media were collected and stored at -20°C for determination of basal LH and FSH secretion. The wells were washed with fresh medium and then treated with 0.1 μ mol GnRH l⁻¹ (Fertagyl: Intervet UK Ltd, Cambridge). Cultures were terminated after 4 h of incubation by collecting the media which was stored at -20°C for determination of GnRH-induced LH and FSH secretion.

Dose-responses for assessment of bioactivities

Doses of 0, 0.4, 2.0, 10.0 and 50.0 μ l per well of steroid-free oLE70–80 and hFF, with or without inhibin depletion, and oLE50, 90, 120 and oPE50, 90, 120, were added randomly to nine independent cultures of rat pituitary cells in quadruplicate wells. Each culture dish contained four control wells that received SFDM only and four wells received 50 μ l SFDM rather than 50 μ l GnRH. This procedure enabled the calculation of LH release in the absence of GnRH stimulation to ensure comparable GnRH responses from one culture to another.

Effect of anti-GnSAF polyclonal antibody

Anti-GnSAF antiserum and a control rat polyclonal antiserum (antiserum raised against serum from three postmenopausal women: anti-PMWS) were mixed gently 1:1 in 100 μ l aliquots with oLE70–80 and hFF for 1 h at 37°C in 24-well dishes on an orbital shaker to determine whether the antiserum previously demonstrated to block GnSAF bioactivity *in vitro* in a range of human, bovine and rat preparations had the same effect (Fowler *et al.*, 2002; P. A. Fowler, C. Price and N. Spears, unpublished). The incubation experiment was repeated three times. The samples were then applied to five separate bioassays at 0.5, 5.0 and 50.0 μ l per well. The use of the anti-PMWS controlled for the effects of increasing serum

concentration in the culture dish wells and the addition of rat IgG to rat pituitary cell monolayers.

Pseudochromatofocusing of inhibin-depleted oLE and hFF

Preliminary purification of oLE70–80 was performed by pseudochromatofocusing using 3.3 g Bakerbond CBXTM media (JT Baker Inc., Phillipsburg, NJ) packed into a HRTM 10/10 column (Amersham Pharmacia Biotech Little Chalfont) as described by Fowler and Price (1997). Volumes of 1.5 ml oLE were equilibrated by desalting 0.75 ml aliquots into 2 ml 2, N-morpholino, ethanesulfonic acid buffer (MES; Sigma-Aldrich Co. Ltd, Poole), pH 5.0, mixed 1:1 with MES buffer, adjusted to pH 5.0, centrifuged at 2000 g for 15 min, and passed through a 0.2 μ m filter before chromatography. The samples were pseudo-chromatofocused at 200 ml h⁻¹. A pH gradient of 5–8.0 was generated by sequentially passing 30 ml of 75% MES + 25% sodium acetate (500 mmol sodium acetate (NaAc) l⁻¹, pH 8.00), 50% MES + 50% NaAc and 25% MES + 75% NaAc through the column. Each run was terminated with 140 ml of 100% NaAc buffer. The pH of each of the fractions was determined before being concentrated to 1 ml through MacrosepTM 10 kDa MWCO filters at 5000 g (Flowgen Instruments Ltd, Sittingbourne). Before the bioassay, each concentrated fraction was passed through a 0.2 μ m filter and double-desalted. The chromatography fractions were bioassayed at 5 μ l per well in two separate rat pituitary cell cultures.

Hormone assays

Concentrations of LH and FSH in cell-conditioned media from rat cultures were determined using homologous rat radioimmunoassays. Sensitivities and intra- and interassay coefficients of variation values of these radioimmunoassays were: 0.6 ng FSH ml⁻¹ (NIDDK-rFSH-RP-2) using NIDDK-anti-rFSH-S11 and 7.1 and 11.2%, respectively, and 0.3 ng LH ml⁻¹ (NIDDK-rLH-RP3) using NIDDK-anti-rLH-S11 and 8.5 and 13.3%, respectively. Commercial kits were used to determine oestradiol and progesterone concentrations in hFF and oLE as described in Fowler *et al.* (1990).

Statistical analysis

The *in vitro* pituitary responses are expressed as percentages of the relevant control gonadotrophin concentrations secreted from blank control wells on the same culture dishes, allowing data from different rat pituitary monolayer cultures to be pooled. The controls were wells exposed to SFDM alone (basal secretion), both overnight and during the 4 h exposure to GnRH, and wells exposed to SFDM + 0.1 μ mol GnRH l⁻¹ (GnRH-induced secretion) during the 4 h GnRH challenge. The differences between treatments and dose-responses

Fig. 1. Dose-dependent effects of steroid-free (a,c,e,g) human follicular fluid (hFF) and (b,d,f,h) ovine luteal extracts at days 70–80 of pregnancy (oLE70–80), both with (●) and without (○) inhibin depletion, on (a,b) GnRH-induced and (c,d) basal LH secretion and on (e,f) GnRH-induced and (g,h) basal FSH secretion from two different rat pituitary gland monolayers. The horizontal shaded bar represents the SEM range for control secretion. Significance values are by ANOVA relative to controls (culture medium only), * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, with the significance of effects of inhibin depletion shown by brackets and P values, $n = 2 \times 4$; plotted values are means $\pm$ SEM.

were assessed by two-way ANOVA. Differences between specific categories were tested by Dennett's *post hoc* test (comparison of tests with control). Median effective doses (ED_{50} value) for GnSAF bioactivity (defined as

the μ l of hFF or extract per well dose required to produce 50% of the greatest suppression of secretion observed, converted to mg of protein) were calculated from the dose–response curves using log-linear regression equations fitted separately for each dose–response curve. The analyses were performed using the Statview 4.02 programme (Abacus Concepts Inc., Berkley, CA). All results are presented as means $\pm$ SEM.

Results

Effects of oLE70–80 hFF on gonadotrophin secretion

All four preparations (steroid-free oLE70–80 and hFF with or without inhibin depletion) significantly suppressed GnRH-induced LH secretion (ANOVA, $P < 0.001$; Fig. 1a,b) by up to $47 \pm 5\%$ and $68 \pm 4\%$ (hFF) and $42 \pm 5\%$ and $49 \pm 11\%$ (oLE70–80), respectively, but had no dose–response effect on basal LH (Fig. 1c,d). Both hFF preparations, but not the oLE70–80 preparations, caused some stimulation of basal LH at the highest dose (by $28 \pm 8\%$ and $18 \pm 6\%$, ANOVA, $P < 0.05$; $P < 0.01$). Inhibin-depleted hFF and oLE70–80 significantly suppressed GnRH-induced FSH secretion (Fig. 1e,f) by up to $50 \pm 10\%$ and $37 \pm 5\%$ (ANOVA, $P < 0.001$). In contrast, only the non-depleted hFF and oLE70–80 suppressed basal FSH in a significant dose-dependent manner, with a maximal suppression of $51 \pm 4\%$ and $36 \pm 5\%$ (ANOVA, $P < 0.001$), respectively. However, inhibin-depleted hFF and oLE70–80 did cause some suppression of basal FSH secretion (by $20 \pm 4\%$ and $25 \pm 8\%$, ANOVA, $P < 0.05$ $P < 0.001$), although this was not in a dose-dependent manner (Fig. 1g,h).

The ED_{50} values for these effects on gonadotrophin secretion (Table 1) showed that inhibin-depleted hFF had less effect on GnRH-induced LH secretion than did steroid-free oLE70–80 or hFF. Inhibin-depleted hFF had the greatest effect on GnRH-induced FSH, whereas only non-depleted hFF and oLE70–80 contained marked inhibin bioactivity in terms of reducing basal FSH secretion.

Effect of rat anti-GnSAF polyclonal on antiserum inhibin-depleted oLE70–80 bioactivity

Incubation of oLE70–80 with either the rat GnSAF-blocking polyclonal antiserum or antiserum raised against serum proteins from post-menopausal women had no effect on the suppression of GnRH-induced LH secretion *in vitro* (Fig. 2b), with GnRH-induced LH secretion $58 \pm 7\%$ and $56 \pm 4\%$ of control, respectively (ANOVA, this result was not significant). However the blocking antiserum clearly blocked GnSAF bioactivity in hFF (Fig. 2a), with GnRH-induced LH secretion at $96 \pm 6\%$ of control in the presence of the blocking antiserum compared with $64 \pm 9\%$ of control in the presence of the non-specific antiserum (ANOVA, $P < 0.001$).

Table 1. Median effective doses (ED₅₀ values) for GnRH-induced LH-suppressing bioactivity and inhibin bioactivity in steroid-free non-depleted and in steroid-free inhibin-depleted hFF and oLE70–80

	Inhibin-depleted, steroid-free preparations		Steroid-free, non-depleted preparations	
	hFF	oLE70–80	hFF	oLE70–80
	ED ₅₀ (mg protein per well)			
GnRH–LH	1.44	0.18	0.11	0.15
GnRH–FSH	0.09	0.24	0.20	0.20
Basal FSH	*	*	0.14	0.26
Basal LH	*	*	*	*

*No significant dose–response effect: ED₅₀ value not calculated.

hFF: human follicular fluid; oLE70–80: ovine luteal extracts at days 70–80 of gestation.

Fig. 2. Dose-dependent effects of steroid-free, inhibin-depleted, human follicular fluid (hFF) and ovine luteal extracts at days 70–80 of pregnancy (oLE70–80) after incubation with anti-gonadotrophin surge-attenuating factor (GnSAF) antiserum (●) or anti-post-menopausal woman serum antiserum (○) on GnRH-induced LH secretion from five different rat pituitary monolayers. The horizontal shaded bar represents the SEM range for control secretion. Significance values are by ANOVA relative to controls (culture medium only), * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, with the significance of effects of antibody treatment shown by brackets and P values, $n = 5 \times 4$; plotted values are means $\pm$ SEM.

Effect of oLE and oPE on GnRH-induced LH secretion

All six preparations (oLE50, 90, 120 and oPE50, 90, 120) significantly reduced GnRH-induced LH secretion to below 60% of control (Fig. 3). However, although the oLE preparations showed only a moderate increase in GnRH-induced LH-suppressing bioactivity as pregnancy continued (ED₅₀ value decreasing from 0.15 mg to 0.10 mg from day 50 to day 120 of gestation, ANOVA, not significant), there was a threefold increase in GnRH-induced LH-suppressing bioactivity in the oPE preparations (ED₅₀ value decreasing from 0.27 mg to 0.09 mg to

Fig. 3. Dose-dependent effects of steroid-free extracts of ovine corpora lutea of pregnancy (ovine luteal extracts (oLE; a,b,c)) and ovine placenta (ovine pituitary extracts (oPE; d,e,f)) collected on (a,d) day 50, (b,e) day 90 and (c,f) day 120 of gestation on GnRH-induced LH secretion from two different rat pituitary monolayers. The horizontal shaded bar represents the SEM range for control secretion. Significance values are by ANOVA relative to controls (culture medium only), * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, $n = 3 \times 4$; plotted values are means $\pm$ SEM.

0.09 mg from day 50 to day 120 of gestation, ANOVA, $P < 0.01$).

Partial purification of inhibin-depleted oLE70–80 GnRH-induced LH-suppressing bioactivity

The pH gradient-driven protein elution profiles for steroid-free, inhibin-depleted oLE (Fig. 4) were similar

Fig. 4. Pseudochromatofocusing resolution of GnRH-induced LH suppressing bioactivity in inhibin-depleted, steroid-free, ovine luteal extracts at days 70–80 of pregnancy. The thin line shows the protein elution profile at 280 nm and the thick line shows the pH of the fractions eluted from the column. GnRH-induced LH secretion (●) and the elution of suppressive bioactivity (○) at pH 5.40 + 5.77. The horizontal shaded bar represents the SEM range for control secretion. $n = 2 \times 4$; plotted values are means $\pm$ SEM.

to those observed for hFF and bovine serum (Fowler and Price, 1997) and for granulosa–luteal cell-conditioned medium (Fowler *et al.*, 2002). The oLE70–80 contained clear bioactivity that suppressed GnRH-induced LH secretion (GnRH-induced LH secretion reduced by $30 \pm 17\%$ and $29 \pm 12\%$, respectively, ANOVA, $P < 0.01$) and eluted at pH 5.40 and pH 5.77 by pseudochromatofocusing.

Discussion

The present study demonstrates conclusively that the ovine corpus luteum of pregnancy contains non-steroidal, non-inhibin bioactivity that reduces pituitary gland responsiveness to GnRH *in vitro*. Extracts of the ovine corpus luteum significantly reduced the release of both LH and FSH from cultured rat gonadotrophs by GnRH. However, this bioactivity did not have dose-dependent effects on basal gonadotrophin secretion. The behaviour of the steroid-free, inhibin-depleted oLE70–80 extract in our established rat pituitary cell culture bioassay, with pI values of 5.40 and 5.77, was markedly similar to that of steroid-free, inhibin-depleted human ovarian GnSAF (pH 5.25 and pH 5.75, Mroueh *et al.*, 1996; pH 5.74 and pH 5.77, Fowler *et al.*, 2002).

Furthermore, preliminary data indicate that serum from women in the third trimester of gestation contains GnSAF bioactivity (Fowler *et al.*, 1995). However, the observation in the present study that a GnSAF antiserum which blocks human GnSAF (Fowler *et al.*, 2002) and bovine and rat GnSAF (P. A. Fowler, C. Price and N. Spears, unpublished), but not oLE70–80 bioactivity, does not support the conclusion that the ovine corpus luteum GnRH-induced LH-suppressing bioactivity is the same as GnSAF.

The removal of almost all of the oestradiol and progesterone from the ovine corpus luteum extracts before bioassay indicates that the bioactivity in ovine corpus luteum extract that suppresses pituitary gland responsiveness to GnRH is distinct from ovarian steroids. In addition, long-term (24 h) exposure of rat gonadotrophs to oestradiol *in vitro* had little effect on GnRH-induced LH secretion (Speight and Fink, 1981; Byrne *et al.*, 1995). Continued exposure to oestradiol during a GnRH challenge augments GnRH-induced LH release and synthesis (Ramey *et al.*, 1987), whereas the co-administration of progesterone with GnRH augments LH release from rat pituitary gland monolayers (Geiger *et al.*, 1980; Leigh *et al.*, 1994). It is reasonable to conclude that the effects of ovine corpus luteum extracts in the present study were not due to the trace quantities of steroid hormones remaining after charcoal treatment.

Although inhibin has marked suppressive effects on FSH secretion, its suppressive effects on LH release from cultured rat gonadotrophs are seen only at higher doses: reducing GnRH-induced LH secretion (Campen and Vale, 1988), gonadotrophin subunit mRNA expression (Attardi *et al.*, 1989, 1991) and GnRH receptor expression (Wang *et al.*, 1989; Braden *et al.*, 1990) or binding (Wang *et al.*, 1988). The present study compared inhibin containing hFF with inhibin depleted of hFF. Inhibin without hFF, unlike inhibin containing hFF, did not suppress basal FSH secretion below 80% of the control value, demonstrating that very little inhibin bioactivity remained. Despite this finding, both preparations reduced GnRH-induced LH secretion to $< 60\%$ of the control value. This finding supports the conclusion that the ovine corpus luteum extract treated in the same way reduced GnRH-induced LH secretion because of some bioactive component other than inhibin. This contention is reinforced by findings that inhibin is reduced by day 90 of gestation in the ewe (Knight *et al.*, 1998), the opposite of the increasing GnRH-induced LH-suppressing effects observed in the present study.

The present study has demonstrated that the ovine corpus luteum of pregnancy produces a non-steroidal, non-inhibin bioactivity which would be able to damp pituitary responsiveness to GnRH during pregnancy. It has been suggested that high steroid hormone concentrations during late pregnancy in the ewe (Thompson and Wagner, 1974; Challis and Patrick, 1981; Thomas *et al.*, 1994) may be responsible for long-term downregulation

of GnRH secretion. Interestingly, administration of oestradiol significantly reverses the long-term downregulation of GnRH receptor expression and binding, but not LH expression, in GnRH agonist-treated ewes (Brooks and McNeilly, 1994). Although there is no data on GnRH pulsatility during gestation in the ewe, regular LH pulsatility is absent in the peri-implantation period (Al-Gubory, 1999) and throughout pregnancy (Al-Gubory *et al.*, 2003). It is also known that in pregnant women circulating GnRH titres decrease during gestation (Siler-Khodr *et al.*, 1984). In addition, although GnRH pulse frequency remains unchanged in pregnant women, GnRH pulse amplitude during late gestation is half that seen in early gestation (Petraglia *et al.*, 1994). Furthermore, ovine pituitary responsiveness to GnRH in the short-term *in vitro* is not a result of decreased GnRH receptor expression after day 45 of pregnancy (Fowler and McNeilly, 1997). By day 60 of pregnancy, at a time when circulating concentrations of progesterone are similar to those measured on day 10 of the oestrous cycle, a marked inhibition of pulsatile LH release and pituitary responsiveness to GnRH has been shown to occur as compared with those on day 10 of the oestrous cycle (Al-Gubory *et al.*, 2003). This finding indicates that sustained high concentrations of progesterone cannot be the sole cause of the inhibition of pulsatile LH secretion. If oestradiol is an important inhibitor of LH pulse amplitude and frequency observed on day 60 and day 120 of pregnancy (Al-Gubory *et al.*, 2003), then circulating concentrations of this steroid should be noticeably increased after mid-pregnancy. This is probably not the case as the concentrations of circulating oestradiol remained very low throughout pregnancy and increased only in the last few days of pregnancy (Tsang, 1978). Therefore, the ability of non-steroidal factors from the ovine corpus luteum of pregnancy, and indeed the placenta, to suppress pituitary responsiveness to GnRH would be important in the prevention of an untimely increase in gonadotrophin secretion and subsequent follicular development.

It is concluded that the corpus luteum of pregnancy in sheep produces a non-steroidal, non-inhibin, bioactivity that suppresses pituitary gland responsiveness to GnRH *in vitro*. Although this bioactivity has some similarities to the putative ovarian hormone GnSAF, it is probably a distinct entity.

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