GnRH-dependent and -independent components of FSH secretion after acute treatment of anoestrous ewes with ovine follicular fluid and a GnRH antagonist

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Gonadotrophin and inhibin concentrations were measured in anoestrous ewes after acute treatment with either saline, ovine follicular fluid (oFF), GnRH antagonist, or oFF and GnRH antagonist in combination. The increase in mean LH concentrations observed in ewes treated with oFF alone, was not seen in either of the groups treated with GnRH antagonist, in which LH pulsatility was completely inhibited. This result suggests that the LH rebound that follows follicular fluid treatment is GnRH dependent. Blockade of GnRH had no effect on the suppression of FSH seen after follicular fluid injection, indicating that this component of FSH secretion is independent of short-term GnRH input. After this initial suppression, a rebound release of FSH was seen in the group treated with oFF alone. The addition of GnRH antagonist appeared to decrease the rebound, suggesting that this rebound release of FSH may have a GnRH-dependent component. Inhibin concentrations in both oFF-treated groups increased after oFF injection and then declined to pretreatment values. However, a second rise in inhibin concentration, concomitant with the FSH rebound in ewes receiving oFF alone, was seen in the group treated with oFF and GnRH antagonist. As this rise in endogenous inhibin concentration could also act to suppress the rebound release of FSH, it cannot be conclusively proved from this study that GnRH input is required for the generation of the rebound release of FSH after treatment with oFF.

Introduction

It is well established that treatment of ewes with follicular fluid as a source of inhibin results in a suppression of FSH secretion followed by hypersecretion of FSH after treatment ceases (Wallace and McNeilly, 1986). This rebound release of FSH has been likened to the secondary FSH peak occurring in the normal cycle and results in increased follicle growth and ovulation in cyclic ewes (Wallace and McNeilly, 1985). These changes in FSH secretion after treatment with bovine follicular fluid have been associated with changes in pituitary content of FSH and FSHβ mRNA and were attributed to lowered oestradiol concentrations resulting from retardation of follicle development (Brooks et al., 1992).

It is not known whether a continued input of GnRH is required to allow generation of this rebound release of FSH. Administration of GnRH antibody had no effect on the appearance of the secondary FSH peak in ewes (Narayana and Dobson, 1979) and failed to block pulsatile FSH secretion in the castrated male rat (Culler and Negro-Vilar, 1987). In addition, the postovulatory surge of FSH in the rabbit was not altered by GnRH antagonist treatment (Mills et al., 1983) and Grady et al. (1985) found only a partial inhibition of FSH secretion after treating ovariectomized rats with a GnRH antagonist. They suggested that there are both GnRH-dependent and GnRH-independent components of FSH synthesis. The GnRH-independent component has been shown to be sensitive to follicular fluid treatment (Charlesworth et al., 1984; Knight and Castillo, 1988). The present study was designed to examine the effect of blocking GnRH action, by use of a GnRH antagonist, on the suppression and rebound release of FSH induced by ovine follicular fluid in anoestrous ewes.

Materials and Methods

Animals and experimental design

Twenty Welsh Mountain ewes were studied during mid-anoestrus in May 1990. Ewes, 4 years old and weighing 34-42 kg, were allocated randomly to one of four treatment groups. On the day before the start of the experiment one jugular vein was cannulated. Animals in groups 1 and 3 received 3 ml (s.c.) saline at 09:00 h on day 1 of the experiment, whereas groups 2 and 4 received 3 ml (s.c.) ovine follicular fluid (oFF). In addition, groups 3 and 4 received GnRH antagonist (Nal-Glu.HOAc, code no. 139-199-30 [Salk Institute], 3 mg s.c. in 0.9% (w/v) saline) at 09:00 h on days 1 and 2. This dose of antagonist has previously been shown to inhibit the pulsatile secretion of LH for at least 24 h (Campbell...
et al., 1990). Samples of jugular venous blood (2.5 ml) for the determination of mean LH, FSH and inhibin concentrations were collected at 2–4 h intervals from −2 h to +56 h during the experimental period. In addition, serial blood samples were taken at 15 min intervals from +24 h to +30 h to allow measurement of the pulsatile secretion of LH.

### Follicular fluid preparation

Ovine FF was aspirated from all visible follicles in sheep ovaries obtained from a local abattoir. The pooled FF was charcoal stripped, centrifuged (1000 g for 30 min), passed through C₄ sep-pak cartridges (Waters Associates, Milford, MA) and stored at −20°C until use, as described by Baird et al. (1990). The FF was shown to have a bioactivity of 8076 IU ml⁻¹ using an ovine inhibin bioassay (Tsonis et al., 1986).

### Hormone assays

Plasma concentrations of ovine FSH, LH and the 1–26 α-subunit of inhibin were measured in duplicate using radioimmunoassays described previously (McNeill et al., 1976; McNeilly and Fraser, 1987; McNeilly et al., 1989). Assay sensitivities were 5.4 ng NIDDK ovine FSH-S14 ml⁻¹, 0.3 ng NIDDK ovine LH-S23 ml⁻¹ and 39.0 pg 1–26 α-subunit ml⁻¹. The intra- and interassay coefficients of variation (CV) were 10.1 and 9.6% for FSH, and 13.5 and 10.6% for LH, respectively. For inhibin, all samples were measured in a single assay; the intra-assay CV was 12%.

### Statistical analysis

Results were analysed by split-plot analysis of variance followed by Newman–Keuls test when a significant (P < 0.05) interaction was found. Plasma hormone data were shown to be positively skewed and therefore were log transformed before analysis to reduce heterogeneity of variation. The parameters of LH pulsatile secretion were determined using the Munro pulse analysis program (Zaristow Software, Haddington, East Lothian), using the Munro algorithm with the following parameters: Baxter parameters h₁ = 0.0141, h₂ = 0.0781, b₃ = 0; G parameters, G₁ = 3.98, G₂ = 2.4, G₃ = 1.68, G₄ = 1.24, G₅ = 0.93. Comparisons between groups on these parameters were made using analysis of variance. All values are reported as means ± SEM, n = 5. For clarity, results for groups 1 and 3, and 2 and 4 are shown on separate graphs, although they were analysed as a whole.

### Results

#### Plasma FSH concentrations

A significant (P < 0.05) decrease in FSH concentrations in plasma was seen in both oFF-treated groups in the 8–24 h after injection compared with groups 1 and 3 (FSH values during this period were 35.6 ± 0.6, 11.4 ± 0.6, 24.5 ± 0.4 and 8.2 ± 0.5 ng ml⁻¹ for groups 1–4, respectively; Fig. 1). Thereafter, FSH concentrations in these two groups returned to control (group 1) values, and a rebound effect was detected in group 2. This rebound release was significantly (P < 0.05) different from the FSH concentrations seen at the time of maximum suppression, but was not different from pretreatment values or the control group at this time. Concentrations of FSH for the period 44–56 h after injection were 38.4 ± 6.4, 58.0 ± 13.9, 22.7 ± 2.2 and 37.1 ± 8.3 ng ml⁻¹ for groups 1–4, respectively. The rebound effect in the ewes treated with oFF and GnRH antagonist appeared to be less than in ewes given oFF alone. Although there was a 36% difference between these two groups, this was not statistically significant. The GnRH antagonist treatment alone appeared to suppress FSH concentrations compared with controls throughout the experimental period, although this was only significant (P < 0.05) at 16–20 h after the first injection of antagonist.

### Concentrations of LH in plasma

Mean LH concentrations for groups 1–4 over the period −2 h to +56 h were 0.97 ± 0.05, 1.94 ± 0.18, 0.58 ± 0.01 and 0.59 ± 0.01 ng ml⁻¹, respectively, and concentration of LH in group 2 was significantly (P < 0.01) higher than in the other groups. Mean LH concentrations in the GnRH antagonist-treated animals (groups 3 and 4) were always lower than controls throughout the experimental period, although they were not significantly different at the experimental time (Fig. 2). Ovine FF treatment alone (group 2) increased mean LH concentrations; concentrations were consistently higher (P < 0.05) than the other groups from 40 to 56 h after oFF injection, and maximum secretion (5.15 ± 2.22 ng ml⁻¹) was seen at 44 h after oFF.
Parameters of pulsatile LH secretion are shown (Table 1). Basal LH secretion was unaffected by the different treatments. Ovine FF treatment alone, however, caused a significant (P < 0.01) increase in the frequency of LH pulses, but did not alter pulse amplitude. GnRH antagonist treatment completely inhibited the pulsatile secretion of LH as no pulses were detected in groups 3 and 4.

### Plasma inhibin concentrations

As expected, a significant (P < 0.05) increase in inhibin concentrations was seen in groups 2 and 4 at 4–16 h after injection of oFF in comparison with groups 1 and 3 (Fig. 3). Concentrations then declined to pretreatment values by 24–28 h in both groups and indeed stabilized at pretreatment values in the animals treated with oFF alone. However, in the animals receiving both oFF and GnRH antagonist treatment, a second significant (P < 0.05) rise in inhibin concentrations was again seen at 36–52 h after injection. No significant change in inhibin concentrations was seen throughout the experimental period in the controls, or in animals treated with GnRH antagonist alone. Inhibin concentrations tended to be lower in group 3, although they were only significantly (P < 0.05) different at 12–16 h after the first injection of GnRH antagonist.

## Discussion

There are now several studies showing a suppression in FSH concentrations after steroid-free follicular fluid treatment in both cyclic (Wallace and McNeilly, 1985; Henderson et al., 1986; Brooks et al., 1992) and seasonally anoestrous ewes (McNatty et al., 1985; McLeod and McNeilly, 1987). The results of the study reported here confirm these findings and also show that the suppression in FSH secretion is independent of short-term GnRH input. This finding complements an earlier study (Knight and Castillo, 1988) in which bovine FF significantly suppressed FSH concentrations both before and after GnRH agonist treatment in anoestrous ewes. It therefore appears that there is a GnRH-independent component of FSH secretion that is sensitive to the inhibitory effects of steroid-free follicular fluid. A rebound release of FSH followed the initial suppression in FSH secretion in the group treated with oFF, which supports previous findings (Wallace and McNeilly, 1985; Brooks et al., 1992). Blocking GnRH action did appear to blunt the FSH rebound, although this was not a significant effect. FSH secretion appears to be less dependent on GnRH input than does LH secretion (McNeilly, 1988). In the short term, FSH concentrations increase in follicular phase ewes following injection of both GnRH antiserum (Fraser and McNeilly, 1983) and GnRH antagonist (Campbell et al., 1990) due mainly to the removal of the negative feedback effects of ovarian steroids and inhibin. Chronic inhibition of GnRH in cyclic ewes by GnRH agonist treatment suppressed FSHβ mRNA concentrations and FSH secretion by only 30–50%, whereas LHβ mRNA and LH secretion were decreased by > 95% (McNeilly et al., 1991). Furthermore, sheep pituitary cells synthesize FSH, but not LH, in vitro in the absence of GnRH input (Miller et al., 1977).

This study has also confirmed our previous findings that follicular fluid treatment causes increased mean LH secretion at the same time as the rebound release of FSH, which has been attributed to reduced oestradiol output from the ovary at this time (Wallace et al., 1988; Brooks et al., 1992). Results from in vitro studies that have used ovine pituitary cell cultures appear to suggest that inhibin may act at the pituitary to modulate LH secretion directly by upregulation of GnRH receptors (Gegg et al., 1991; Mutukrishna and Knight, 1990). However, in vivo studies have not been able to repeat these findings (Brooks et al., 1992).

It is well established that oestradiol secretion is under the acute positive regulation of LH (Baird and McNeilly, 1981). Indeed, an immediate inhibition of the pulsatile secretion of oestradiol has been found following GnRH antagonist treatment in follicular phase ewes with ovarian autotransplants (Campbell et al., 1990). In this study, a concomitant increase in FSH concentrations was also seen owing to the removal of the negative feedback effects of oestradiol (Campbell et al., 1990). However, in the present study using anoestrous ewes, a decrease in FSH concentrations was seen after GnRH antagonist treatment. Like LH, FSH also shows a seasonal change in responsiveness to the negative feedback effects of oestradiol (Legan and Kirsch, 1980). Nevertheless, as oestradiol concentrations in anoestrous ewes are already minimal (Goodman et al., 1981), it is possible that under these circumstances the decline in oestradiol following GnRH antagonist treatment is not the primary factor in determining the amount of FSH secretion. Thus, the decrease in FSH concentrations seen in the ewes treated with GnRH antagonist alone may be a direct consequence of blocking GnRH input.

The finding that combined treatment with oFF and GnRH antagonist acted to increase endogenous inhibin secretion in the
Table 1. Changes in basal concentration, pulse frequency and pulse amplitude of LH 24–30 h after first injection in untreated ewes and ewes treated with ovine follicular fluid or GnRH antagonist (GnRH-A) alone or in combination during mid-anoestrus.

<table>
<thead>
<tr>
<th>Group</th>
<th>Basal LH (ng ml⁻¹)</th>
<th>LH pulses (number in 6 h)</th>
<th>LH pulse amplitude (ng ml⁻¹)</th>
<th>Number of animals showing LH pulses</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (control)</td>
<td>0.63 ± 0.08</td>
<td>0.4 ± 0.3*</td>
<td>3.06</td>
<td>2/5</td>
</tr>
<tr>
<td>2 (oFF)</td>
<td>0.90 ± 0.23</td>
<td>2.4 ± 0.7b</td>
<td>2.31 ± 0.59</td>
<td>5/5</td>
</tr>
<tr>
<td>3 (GnRH-A)</td>
<td>0.58 ± 0.06</td>
<td>0*</td>
<td>—</td>
<td>0/5</td>
</tr>
<tr>
<td>4 (oFF + GnRH-A)</td>
<td>0.55 ± 0.07</td>
<td>0*</td>
<td>—</td>
<td>0/5</td>
</tr>
</tbody>
</table>

Values are means ± SEM, n = 5. Data were analysed by one-way analysis of variance.

Fig. 3. Changes (mean ± SEM, n = 5) in concentration of inhibin in (a) mid-anoestrus ewes left untreated (Group 1, ○) or injected with GnRH-antagonist (Group 3, ●); and (b) mid-anoestrus ewes injected with oFF alone (Group 2, □) or oFF plus GnRH-antagonist (Group 4, ■). Blood samples were taken every 4 h. Log-transformed data were analysed by two-way analysis of variance. Symbols denote significant differences between groups (P < 0.05) as follows: *G2 and G4 different from G1 and G3; †G2 and G4 different from G3; ‡G2 different from G4; ‡G1 different from G3.

The present study was unexpected. One possible explanation is that the increase in inhibin was a consequence of the inhibition of LH pulsatility by the GnRH antagonist treatment which modulated the follicle growth induced by the rebound release of FSH. As discussed previously, there was an increase in LH pulse frequency in the animals treated with oFF alone. Previous work has shown that LH pulses are important in the follicle selection process and that high amplitude LH pulses may act to interfere with FSH-induced follicle development (Picton et al., 1990; McNeilly et al., 1992). Thus, in the animals given the combined oFF and GnRH-antagonist treatment, in which no LH pulses were seen, it is possible that there would be no restraint on follicle growth, resulting in increased inhibin production. The increase in endogenous inhibin concentrations seen after oFF and GnRH antagonist treatment in the present study at the time of minimal LH secretion also confirms earlier findings (Campbell et al., 1989) that inhibin is not under the acute stimulatory control of LH in anoestrous ewes.

In conclusion, this study has found that a single injection of oFF to anoestrous ewes caused an initial suppression of FSH secretion which was not affected in the short term by blockade of GnRH. This result suggests that this component of FSH secretion is independent of GnRH. In contrast, the rebound release of FSH that follows this initial suppression may have a GnRH-dependent component. It is difficult, however, to draw definite conclusions as the rise in inhibin concentrations seen at this time may also act to decrease concentrations of FSH.

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