Examination of the relative role of FSH and LH in the mechanism of ovulatory follicle selection in sheep

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The GnRH-agonist suppression–ovarian autotransplant model (n = 18) was used to examine the relative roles of temporal changes in FSH and LH stimulation on follicle development and selection. Follicle development was stimulated by infusion of FSH for 3 days and treatments applied for 60 h after progestagen sponge withdrawal and before delivery of an ovulatory stimulus. In Expt 1, there was continuous infusion of FSH with or without small amplitude high frequency LH pulses, or withdrawal of FSH with or without pulsatile LH. In Expt 2, there was acute or gradual withdrawal of FSH at sponge withdrawal with pulsatile LH. The patterns of follicle development and basal and pulsatile ovarian hormone secretion were determined. The maintenance of FSH throughout the artificial follicular phase resulted in multiple follicle development and ovulation (3.2 ± 0.62). Pulsatile LH stimulated steroid secretion (P < 0.001) but had little effect on ovulation rates (3.2 ± 0.8) when FSH was maintained. However, withdrawal of FSH in the absence of LH resulted in atresia of the ovulatory follicles and anovulation whereas, when FSH was withdrawn in the presence of LH, preovulatory follicle development was maintained in some animals (3/6 and 5/9 in Expts 1 and 2, respectively) and these ewes had lower (P < 0.05) ovulation rates (1–2 ovulations per ewe). When FSH was withdrawn gradually in the presence of pulsatile LH, 9/9 animals ovulated with ovulation rates in the normal range. These results indicate that ovulatory follicles can transfer their gonadotrophic dependence from FSH to LH. It is hypothesized that the ability of a follicle to respond to this switch in gonadotrophic support is central to the mechanism of follicle selection.

Introduction

The number of oocytes ovulated each cycle is regulated precisely in humans and domestic ruminants and rarely falls outside 1–3. Although the exact mechanisms regulating the selection of ovulatory follicles from a much larger follicular cohort remain obscure, it is clear that selection occurs during the follicular phase when FSH concentrations are falling and LH pulse frequency is increasing (for reviews, see Baird 1983; Scaramuzzi et al., 1993). However, the relative importance of these endocrine changes to the process of follicular selection is unclear.

There is a large body of data supporting a pivotal role for FSH in the control of follicular development (McNeilly et al., 1991). For example, FSH alone (Picton et al., 1990a), but not LH alone (Picton et al., 1990b), can stimulate the growth of follicles to a preovulatory size in ewes made hypogonadotrophic by long-term treatment with the GnRH agonist, buserelin. Baird (1983) hypothesized that the ability of a follicle to withstand the depression in FSH that occurs during the follicular phase is the central mechanism of follicle selection and dominance. However, while it is well established, direct evidence for this hypothesis is relatively scarce as it is difficult to depress FSH acutely and specifically. Experiments that use follicular fluid (McNeilly 1984; Henderson et al., 1986; Baird et al., 1990; Campbell et al., 1991a) or inhibit (Campbell et al., 1996) to depress FSH during the follicular phase in sheep are confounded by potential local actions of inhibin (Hillier et al., 1991; Campbell and Webb 1995) or other components of follicular fluid (Campbell et al., 1991a, 1996; Hunter et al., 1992; Monget and Monniaux 1995). Furthermore, attempts to use the GnRH-agonist model to examine the role of FSH in the control of follicular development have yielded equivocal results due to the slow decrease in FSH when FSH treatment ceases in GnRH-agonist treated animals (McNeilly et al., 1992).

Although LH is thought of primarily as a steroidogenic...
hormone, it is also necessary for the development and maintenance of antral follicles. In sheep, the final maturation and development of antral follicles to ovulation after luteolysis is dependent on an increase in the pulsatile secretion of LH (Baird and McNeilly, 1981). Furthermore, ovulation can be induced in anoestrous ewes by exogenous administration of hCG, GnRH or LH (Goodman and Karsch, 1980; McNatty et al., 1981; McLeod et al., 1982; McNeilly et al., 1982) or the increase in the release of endogenous LH that results from exposure to rams (Martin and Scaramuzzi, 1983). In addition, LH administered as a series of relatively high amplitude low frequency pulses can inhibit FSH-stimulated follicle development in GnRH agonist-suppressed ewes (Picton et al., 1990b), and immunoneutralization of basal LH by co-administration of LH antiserum also inhibits FSH-stimulated follicle development (Picton and McNeilly, 1991). Taken together, these observations indicate that both FSH and LH play a role in the control of selection of ovulatory follicles and their development to ovulatory size, although the exact mechanisms are unclear.

A model has been developed that uses a GnRH antagonist to suppress endogenous gonadotrophin secretion chronically in ewes with an ovarian autotransplant (Campbell et al., 1998a). The antagonist has the advantage of allowing acute suppression of endogenous LH, and the autotransplant facilitates the determination of morphological and functional follicular responses to exogenous gonadotrophic stimuli. The present study uses this model to determine whether it is the changes in FSH, LH, or a combination of both, that is central to the mechanism of ovulatory follicle selection and development.

Materials and Methods

Experimental animals

The experimental animals were either Border Leicester Merino crossbred (Expt 1; n = 18) or Scottish Blackface Merino crossbred (Expt 2; n = 18) ewes in which the left ovary and its vascular pedicle had been autotransplanted to a site in the neck (Goding et al., 1967) at least 12 months previously with the right ovary being removed at the same time. Both these crosses have similar ovulation rates (1–2) and hormonal profiles (Campbell et al., 1994; Souza et al., 1997a,b). The experiments were conducted during the mid- breeding season; Expt 1 was performed at CSIRO Prospect, Sydney, Australia, and Expt 2 at the Marshall Building, University of Edinburgh, UK. Before the start of intensive blood sampling, the animals were grazed normally and their anoestrous condition during the GnRH-antagonist suppression period was verified by inclusion of a raddled ram in the flock (Radford et al., 1960).

On the day before the start of frequent blood sampling, all animals had a Silastic cannula inserted into the jugular vein cranial to the ovarian jugular venous anastomosis and a further two Silastic cannulae inserted into the contralateral jugular vein as described by Campbell et al. (1998a). After cannulation, the animals were placed in metabolism crates in temperature controlled rooms (20°C) under natural lighting and fed a maintenance diet. The animals had been habituated to the housing conditions and frequent handling before the start of the experiment.

Hormone preparations

The FSH used was NIADDK-oFSH-16, which has a biological potency of 20 U mg⁻¹; one unit has an activity of 1 mg NIH-FSH-S1 and LH contamination of 0.04 × NIH-LH-S1 mg⁻¹. The LH used for pulsatile stimulation was NIADDK-ol.H-26, which has a biological potency of 2.3 U mg⁻¹; one unit has an activity of 1 mg NIH-LH-S1 and FSH contamination of < 0.5% by weight. The LH used for an ovulatory stimulus in Expt 1 was GnP3 R3-5, which has a biological potency of 1.2 U mg⁻¹ and FSH contamination of < 1.0% by weight (Dobson et al., 1997a), whereas ol.H-26 was used in Expt 2. All gonadotrophin preparations were dissolved in 0.9% (w/v) sterile saline with 1% (v/v) normal sheep plasma. The FSH was infused continuously i.v. via one of the small jugular cannulae using Harvard infusion pumps. The LH was administered i.v. via the jugular cannula on the ovarian side, in a pulsatile fashion using Harvard infusion pumps connected to a timer (2 min infusion at a rate of 1 ml min⁻¹). The GnRH antagonist (GnRHa; [AcDNa1L, DCpa2,DTrp3,DArg6,DAIalO] GnRH; donated by the Salk Institute and Centre for Population Research, NICHD, La Jolla, CA) was dissolved in 0.9% (w/v) sterile saline to a concentration of 1 mg ml⁻¹ and administered s.c. (Campbell et al., 1990a). Progestagen pessaries (medroxyprogesteroneacetate; Repromap; Upjohn Pty Ltd, Rydalmere, NSW) were inserted intravaginally and changed every 10–12 days.

Experimental design

Suppression period. A hypogonadotrophic hypogonadal state was induced in all animals by combined 3 week treatment with GnRHa (50 pg kg⁻¹ s.c.) at 4–6 day intervals, and progestagen as described by Campbell et al. (1998). During the experimental period, the same dose of GnRHa was given but the interval between treatments was decreased to every 3–4 days. Progestagen sponges were removed 3 days after the start of FSH infusion (Fig. 1).

Experiment 1. The experimental design was a 2 × 2 factorial; the first factor was two different patterns of FSH administration and the second factor two different patterns of LH administration. The first pattern of FSH consisted of constant infusion of FSH (5 µg h⁻¹) for 5.5 days, while the second pattern involved infusion of FSH at the same rate for the first 3 days only (Fig. 1). The patterns of LH were either no LH (with the exception of daily challenges necessary to evaluate the steroidogenic potential of the follicle population) or a pulsatile pattern designed to mimic the luteal to follicular phase transition, which consisted of a high amplitude pulse (2.5 µg oLH-26) every 4 h for the initial 3 days of FSH infusion then, from the time of sponge withdrawal, a high amplitude pulse (2.5 µg oLH-26) every 3 h for 0.5 days, a low amplitude pulse (1.25 µg oLH-26) every 2 h for 0.5 days and then hourly low amplitude LH
Gonadotrophic regimens, designed to mimic components of the luteal to follicular phase transition, were applied to ewes with an autotransplanted ovary made hypogonadal by treatment for 3 weeks with GnRH antagonist (50 µg kg⁻¹ per 96 h) and progestagen sponges (n = 18). Large antral follicle development was stimulated by infusion of 5 µg oFSH-16 h⁻¹ (i.v.) for 3 days. The following treatments were applied for 2.5 days after sponge withdrawal: group 1: continuous infusion of FSH (5 µg hr⁻¹) with no LH (n = 4); group 2: continuous FSH (5 µg hr⁻¹) with low amplitude LH pulses (1.3–2.5 µg oLH-S26) of increasing frequency from one pulse per 3 h to one pulse per h (3,2,1 regimen; n = 4); group 3: withdrawal of FSH with no LH (n = 4); group 4: withdrawal of FSH with 3,2,1 LH (n = 6). Sixty hours after sponge withdrawal, an ovulatory stimulus (8 h infusion of 150 µg GnP3 R3–5 LH h⁻¹ i.v.) was applied. Samples of ovarian and jugular venous blood were collected every 3–4 h over the experimental period, and there were five periods of intensive blood sampling (15 min intervals for 2.5 h at −2, −1, 0, 1 and 2 days after sponge withdrawal; ■) when the steroidogenic capacity of the follicles in all 18 ewes was tested around an LH challenge (1.3–2.5 µg, i.v.). The follicle population was determined by daily ultrasonography.

Fig. 1. Schematic representation of experimental design. Gonadotrophic regimens, designed to mimic components of the luteal to follicular phase transition, were applied to ewes with an autotransplanted ovary made hypogonadal by treatment for 3 weeks with GnRH antagonist (50 µg kg⁻¹ per 96 h) and progestagen sponges (n = 18). Large antral follicle development was stimulated by infusion of 5 µg oFSH-16 h⁻¹ (i.v.) for 3 days. The following treatments were applied for 2.5 days after sponge withdrawal: group 1: continuous infusion of FSH (5 µg hr⁻¹) with no LH (n = 4); group 2: continuous FSH (5 µg hr⁻¹) with low amplitude LH pulses (1.3–2.5 µg oLH-S26) of increasing frequency from one pulse per 3 h to one pulse per h (3,2,1 regimen; n = 4); group 3: withdrawal of FSH with no LH (n = 4); group 4: withdrawal of FSH with 3,2,1 LH (n = 6). Sixty hours after sponge withdrawal, an ovulatory stimulus (8 h infusion of 150 µg GnP3 R3–5 LH h⁻¹ i.v.) was applied. Samples of ovarian and jugular venous blood were collected every 3–4 h over the experimental period, and there were five periods of intensive blood sampling (15 min intervals for 2.5 h at −2, −1, 0, 1 and 2 days after sponge withdrawal; ■) when the steroidogenic capacity of the follicles in all 18 ewes was tested around an LH challenge (1.3–2.5 µg, i.v.). The follicle population was determined by daily ultrasonography.
Progestagen pessaries were withdrawn from all animals 3 days after the start of FSH infusion to mimic the luteal to follicular phase transition, and all animals received an ovulatory stimulus 60 h after sponge withdrawal (the normal time taken to induce an LH surge in these animals) in the form of an 8 h infusion of LH (180 mU GnP3 R3·5 h−1 i.v.).

In summary, the animals were allocated randomly to one of four treatment groups as follows (Fig. 1): group 1: continuous FSH–no LH (FSH for 5.5 days with no pulsatile LH; n = 4); group 2: continuous FSH–3,2,1 LH (FSH for 5.5 days with ‘normal’ pulsatile LH patterns; n = 4); group 3: FSH withdrawal–no LH (FSH for the first 3 days with no pulsatile LH; n = 4); and group 4: FSH withdrawal–3,2,1 LH (FSH for the first 3 days with ‘normal’ pulsatile LH patterns; n = 6).

Experiment 2. This experiment tested the effect of acute or gradual withdrawal of FSH at the time of sponge removal. The same suppression regimen and gonadotrophin preparations were used as described for Expt 1, with the exception that oLH-26 was used to provide an ovulatory stimulus (180 mU h−1 i.v.). There were two treatment groups, and the first group (group A) was identical to group 4 of Expt 1 (that is, having treatment consisting of acute withdrawal of FSH at sponge withdrawal with 3,2,1 LH pulses; n = 9) and the second group (group B) had the same pattern of LH stimulation but with a gradual decrease in FSH during the first 24 h of the induced ‘follicular phase’ (n = 9). Thus, in these animals, FSH was infused at a rate of 3.75 μg h−1 from 0 to 8 h, 2.5 μg h−1 from 8 to 16 h and 1.25 μg h−1 from 16 to 24 h after sponge withdrawal (that is, 75, 50 and 25% of the infusion rate used to induce follicular development). Thereafter, the animals in the two experimental groups received exactly the same pattern of gonadotrophin stimulation.

Collection of blood samples

In Expt 1, ovarian (5 ml) and jugular (3 ml) venous blood samples were taken every 3–4 h from the start of FSH infusion (3 days before sponge withdrawal) until 92 h after sponge withdrawal. In Expt 2, samples were collected every 8 h for the first 3 days of FSH infusion and every 4 h for the period after sponge withdrawal. In Expt 1, there were five periods during which more frequent blood samples were collected at 15 min intervals for 15 min before and 2 h after an LH challenge designed to stimulate ovarian steroid secretion. These periods were 24, 48 and 72 h after the start of FSH infusion (follicle growth phase) and 24 and 48 h after sponge withdrawal (follicular phase). In Expt 2, the first two periods were omitted and, therefore, frequent samples were collected just before (–4 to –2 h) and 24 and 48 h after sponge withdrawal. In experimental groups receiving LH, monitoring samples (that is, those collected every 3–4 h) of ovarian venous blood were collected 20–30 min after LH was administered, as this is the time taken for maximum steroidogenic responses to LH stimulation to be reached. Blood was centrifuged at 1.5 g for 20 min at 4°C and the plasma was stored at −20°C.

Ovarian scanning procedure

The diameter of the antral cavity and position of all follicles > 2 mm in diameter in the medial-lateral, dorsal-ventral and cranial-caudal planes were determined each day, as described by Campbell et al. (1998) and Souza et al. (1997a,b). Animals were scanned 6 days after the artificial LH surge for the presence of corpora lutea so that the ovulation rate could be estimated (Souza et al., 1997b).

Hormone assays

Plasma concentrations of LH, FSH, androstenedione (Campbell et al., 1994), immunoreactive inhibin (McNeilly et al., 1989), oestradiol and progesterone (Campbell et al., 1994) were determined in Expt 1 using radioimmunoassays. In Expt 2, the same assays were used for LH and FSH but different antisera were used for oestradiol and progesterone (Campbell et al., 1990b). The sensitivity of the assays for LH, FSH, androstenedione, inhibin, oestradiol and progesterone for Expts 1 and 2, respectively, were 0.2 and 0.2 μg LH l−1, 0.1 and 0.2 μg FSH l−1, 70 pmol androstenedione l−1, 35 ng I-26 α-subunit pig inhibin l−1, 55 and 50 pmol oestradiol l−1 and 380 and 380 pmol progesterone l−1. The intra- and interassay coefficients of variation for all the immunoassays were < 12% in the 20–80% effective dose range.

Statistical analysis

Statistical analysis of hormonal profile data was performed by repeated sample analysis of variance on untransformed (FSH) or log-transformed (LH, inhibin, oestradiol and androstenedione) data partitioned on the basis of treatment, time and treatment × time interaction (ANOVA) using the Systat Statistical Analysis Package (Systat Inc., Evanston, IL) on an Apple Macintosh PowerPC. Data from before and after sponge withdrawal were analysed separately. The steroidogenic response to an LH challenge was determined by calculating the overall mean concentration of oestradiol and androstenedione over the periods of intensive sampling before analysis by repeated sample ANOVA. The effects of treatment on follicle numbers and follicle diameters were determined by repeated sample ANOVA, while treatment effects on progesterone concentrations and ovulation rate were determined by one-way ANOVA with Tukey’s test. The number of follicles and ovulation rates were subjected to (x + 0.5) transformation before analysis.

Results

Initial 3 days of FSH infusion

In Expts 1 and 2, the results of the initial 3 days of FSH infusion are similar to those reported earlier (Campbell et al., 1998) and will be described only briefly here. GnRH antagonist treatment resulted in a 60% decrease (P < 0.01) in FSH concentrations, while LH concentrations decreased below the detection limit of the assay. Follicle development...
Table 1. Mean (± SEM) number of large follicles (≥3.5 mm), mean follicle diameter, ovulation rate (estimated 6 days after ovulatory stimulus) and jugular venous progesterone concentrations (4-6 days after ovulatory stimulus) in GnRH-antagonist suppressed ewes that were stimulated with FSH for 3 days (from day -3 to day 0) and then subjected to a 2.5 day artificial follicular phase initiated by progestagen sponge withdrawal (day 0) and concluded by an ovulatory stimulus.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group 1 (n = 4)</th>
<th>Group 2 (n = 4)</th>
<th>Group 3 (n = 4)</th>
<th>Group 4a (n = 3)</th>
<th>Group 4b (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of large follicles (≥3.5 mm)</td>
<td>3.3 ± 0.8a</td>
<td>4.0 ± 0.9a</td>
<td>2.0 ± 0.4a</td>
<td>2.7 ± 0.9a</td>
<td>3.0 ± 1.6a</td>
</tr>
<tr>
<td>Significance time effect</td>
<td>0.90</td>
<td>0.01</td>
<td>0.04</td>
<td>0.10</td>
<td>0.56</td>
</tr>
<tr>
<td>Follicle diameter (mm)</td>
<td>4.1 ± 0.3a</td>
<td>4.1 ± 0.2a</td>
<td>3.7 ± 0.3a</td>
<td>3.6 ± 0.3a</td>
<td>3.6 ± 0.3a</td>
</tr>
<tr>
<td>Significance time effect</td>
<td>0.06</td>
<td>0.09</td>
<td>0.03</td>
<td>0.48</td>
<td>0.01</td>
</tr>
<tr>
<td>Ovulation rate</td>
<td>3.3 ± 0.3a</td>
<td>3.8 ± 0.8a</td>
<td>0b</td>
<td>0b</td>
<td>1.7 ± 0.3a</td>
</tr>
<tr>
<td>Progesterone concentrations (nmol L⁻¹)</td>
<td>46.9 ± 6.0a</td>
<td>44.7 ± 6.7a</td>
<td>0.10 ± 0.01b</td>
<td>0.4 ± 0.2b</td>
<td>8.9 ± 1.0c</td>
</tr>
</tbody>
</table>

Group 1: continuous FSH + no LH (FSH for 5.5 days with no pulsatile LH; n = 4); group 2: continuous FSH + 3.2,1 LH (FSH for 5.5 days with 'normal' pulsatile LH patterns; n = 4); group 3: FSH withdrawal + no LH (FSH for the first 3 days with no pulsatile LH; n = 4); and group 4: FSH withdrawal + 3.2,1 LH (FSH for the first 3 days with 'normal' pulsatile LH patterns; n = 6).

*Relative to start of FSH infusion. Group 4 is divided into two sub-groups: group 4a, consisting of animals that did not ovulate in response to the LH surge, and group 4b, consisting of animals that did ovulate in response to the LH surge.

(24) Comparisons within treatment groups with time after initiation of artificial follicular phase (that is, within a column).

(25) Indicate significant (P < 0.05) differences between treatment groups on a particular day (that is, within a row).

did not proceed beyond a diameter of 3 mm in these animals. Infusion of FSH for 3 days resulted in a 60% increase (P < 0.001) in FSH concentrations and stimulated the development of large antral follicles (Table 1) and a coincident increase in ovarian secretion of androstenedione, inhibin and oestradiol in all groups (Figs 2-4; P < 0.01). In Exp 1, LH pulses had no effect on the growth rate and number of antral follicles stimulated by FSH (Table 1) but, in the absence of LH, basal steroid secretion was low (<1 ng min⁻¹; Figs 2-3; P < 0.001). However, LH challenges on days 1,2 and 3 revealed no difference in the steroidogenic capacity of the follicle population in any experimental group at any time point (Figs 2 and 3).

Experiment 1a: Continuous FSH with and without pulsatile LH

FSH. Continued infusion of FSH after sponge withdrawal resulted in the maintenance of FSH concentrations for the next 60 h (0.84 ± 0.02; mean ± SEM). Pulsatile LH (group 2) had no effect on the concentration from 0 to 60 h (0.77 ± 0.03) or the profile of FSH during the follicular phase (Fig. 2). The provision of an ovulatory stimulus by infusion of high doses of oLH from 60 to 68 h after sponge withdrawal resulted in peak values of 1.2 ± 0.1 µg FSH L⁻¹ (n = 18; P < 0.01) in all experimental animals (Fig. 2). The magnitude of this induced surge was lower than spontaneous FSH surges observed previously in these animals (Campbell et al., 1994).

Pulsatile LH and ovulatory stimulus. With the exception of the induced LH surge, LH values in group 1 animals remained at the detection limit of the assay (0.2 µg L⁻¹) whereas, in group 2, pulsatile LH infusion resulted in an increase (P < 0.001) in LH to values of 1.1 ± 0.1 µg L⁻¹ (n = 10) at the time of sponge withdrawal (Fig. 2). However, there were no significant treatment effects on the characteristics of induced LH pulses (determined during periods of intensive blood sampling) or on the artificial LH surge and, as a result, these data were pooled across all groups. Injection of 2.5 µg oLH before sponge withdrawal resulted in pulses with an amplitude of 3.5 ± 0.5 µg L⁻¹ superimposed on a baseline of 0.2 ± 0.0 µg L⁻¹ with an overall mean (± SEM) of 0.7 ± 0.1 µg L⁻¹ (n = 18). During the initial 24 h of the follicular phase, LH concentrations decreased (P < 0.05; Fig. 2) and intensive sampling showed that this decrease was a consequence of the decrease in LH pulse amplitude, with a single injection of 1.25 µg oLH 20 h after sponge withdrawal resulted in a pulse of 1.2 ± 0.4 µg L⁻¹ amplitude superimposed on a baseline of 0.2 ± 0.0 µg L⁻¹ to give an overall mean (± SEM) of 0.4 ± 0.1 µg L⁻¹ (n = 18; P < 0.05 compared with 0 h). At 48 h after sponge withdrawal, two injections of 1.25 µg oLH gave two pulses with an amplitude of 1.8 ± 0.5 µg L⁻¹ superimposed on a baseline of 0.2 ± 0.0 µg L⁻¹ to give an overall mean (± SEM) of 0.6 ± 0.1 µg L⁻¹ (n = 18). Infusion of 180 mU oLH h⁻¹ for 8 h resulted in peripheral concentrations of 98 ± 3 µg L⁻¹ (n = 18). The magnitude and duration of the artificial LH surges were similar to those of spontaneous preovulatory surges in these animals (Campbell et al., 1994; Souza et al., 1997a).

Follicle development and ovulatory response. At the time of sponge withdrawal, each animal had developed 3-4 large antral follicles in response to FSH infusion (Table 1). Continued FSH treatment in the absence of LH (group 1) had no effect on the number of large antral follicles, but follicle diameter tended (P = 0.06) to increase across the follicular phase. The combination of FSH with LH pulses (group 2) stimulated an increase in the number of large antral follicles (P < 0.05) with a similar tendency for an increase (P = 0.09) in
Fig. 2. Expt 1: jugular venous FSH (circles) and LH (triangles) (a) and ovarian venous oestradiol (b), androstenedione (c) and inhibin (d) concentrations in response to continuous infusion of oFSH-16 (■) for a total of 5.5 days with either no LH (group 1, open symbols) or pulsatile LH (group 2, closed symbols). The insets show the secretory response to LH challenges (arrowheads) given to both groups of ewes (the scale on the y-axis is the same as for the main figure). The vertical dashed line indicates the time of progestagen sponge withdrawal and the vertical shaded bar indicates the time of the artificial LH surge. Values are means ± SEM.
follicle diameter across the follicular phase. However, between treatment groups, LH pulses had no effect on the follicle diameter or the number of follicles (3–4) that subsequently ovulated in response to the artificial LH surge (Table 1). On the basis of peripheral progesterone measurements, the formation of the resulting corpora lutea was normal, but the high values (> 40 nmol l⁻¹; Table 1) supported the ultrasonography data that the ovulatory response to both these gonadotrophin regimens was greater than normal.

Steroid production. In animals receiving FSH alone (group 1), basal steroid concentrations were low (< 1 nmol l⁻¹) throughout the experimental period except for a transient increase at the beginning of the artificial LH surge (Fig. 2). In contrast, ovarian steroid secretion in ewes that received FSH and pulsatile LH (group 2) was tenfold higher (P < 0.001) than it was in group 1 animals (Fig. 2). In a pattern highly analogous to a normal follicular phase, this LH regimen resulted in a three- to fourfold increase (P < 0.05) in steroid secretion over the ‘follicular phase’ followed by an abrupt decrease (P < 0.001) coincident with the LH surge (Fig. 2). However, these peak values were three- to fourfold higher than normal (Campbell et al., 1994). Despite low basal steroid secretion in group 1 animals, daily LH challenges showed that the follicle population was highly steroiogenic and that there was no difference between groups 1 and 2 in the magnitude of the steroiogenic response to an LH challenge during the follicular phase (Fig. 2 insets). However, within groups, pulsatile oestradiol and androstenedione secretion increased with time in group 2 (P < 0.05), but not group 1, animals across the follicular phase.

Inhibin. In contrast to the ovarian steroids, immunoreactive inhibin secretion was unaffected by pulsatile LH and there was no difference between groups 1 and 2 during the follicular phase and post-surge period (Fig. 2). However, within groups, immunoreactive inhibin concentrations increased (P < 0.01) with time over the follicular phase in group 2 animals but remained stable in group 1 ewes.

Experiment 1b: FSH withdrawal with and without pulsatile LH

FSH. Stopping FSH infusion at the time of sponge withdrawal (groups 3 and 4) resulted in a rapid decrease (P < 0.001) in peripheral FSH concentrations within 12–20 h (0.25 ± 0.02; Fig. 3) in both groups 3 and 4. FSH concentrations remained stable for the remainder of the follicular phase in group 4 ewes (with LH) but increased (P < 0.05) between 36 and 60 h after sponge withdrawal in group 3 (no LH) animals. Despite this increase, the FSH concentration in group 1 and 2 animals was higher (P < 0.01) than in group 3 and 4 animals over the follicular phase.

Pulsatile LH and ovulatory stimulus. The pattern and concentration of LH in groups 3 and 4 were identical to those observed for groups 1 and 2, respectively, as described above (data not shown for clarity in Fig. 3).

Follicle development and ovulatory response. As in groups 1 and 2, at the time of sponge withdrawal, animals in groups 3 and 4 had grown two to three large antral follicles. In group 3 animals, stopping FSH with no LH pulses resulted in a decrease in the number of ovarian follicles (P < 0.05) with none of the animals in this group ovulating and establishing a corpus luteum in response to the artificial LH surge (Table 1). In contrast, the ovulatory response of the six animals that received the 3,2,1 pulsatile LH regimen after withdrawal of FSH (group 4) could be divided into two equal sub-groups. The data for these sub-groups are presented separately. In the first sub-group (group 4a; n = 3), none of the animals ovulated in response to the LH surge, and the number of large follicles tended to decrease (P = 0.1) across the artificial follicular phase (Table 1). In contrast, animals in the second sub-group (group 4b; n = 3) maintained the number of follicles, follicle diameter increased (P < 0.05) during the artificial follicular phase and ovulation occurred in response to the artificial LH surge. There was no evidence of recruitment of additional follicles over the artificial follicular phase. In contrast to the animals that received FSH for the entire follicular phase (groups 1 and 2), the animals that ovulated when FSH was replaced with low amplitude high frequency LH pulses in group 4b had ovulation rates (1–2) and subsequent peripheral progesterone values in the normal physiological range (5–10 nmol l⁻¹), indicating that some degree of follicular selection had taken place (Table 1).

Steroid production. In group 3 animals, basal steroid secretion was low (< 1 nmol l⁻¹) and withdrawal of FSH had no effect on basal steroid secretion (Fig. 3). However, more frequent sampling around an LH challenge revealed a marked increase in the pulsatile steroid response to an LH challenge with time (P < 0.01; Fig. 3 insets). There were no significant differences between animals in sub-groups 4a and 4b in FSH, oestradiol or androstenedione concentrations during the initial 3 days of FSH infusion (Fig. 3). After sponge withdrawal, ovarian steroid secretion fell transiently at 4 and 8 h in both sub-groups but, by 12 h after sponge withdrawal, it had recovered. However, from this point, the endocrine profiles of the two sub-groups differed; steroid concentrations remained stable in ovulatory animals (group 4b) but decreased (P < 0.05) in non-ovulatory animals (group 4a). Basal steroid concentrations in animals in group 4a differed from those of animals in group 3 and group 4b by the time of the artificial LH surge (P < 0.05) but did not differ from those of group 2 animals (Fig. 3). Animals in group 4b exhibited the normal rapid decrease (P < 0.001) in steroid secretion in response to the artificial LH surge.

Inhibin. In group 3, withdrawal of FSH resulted in a decrease in ovarian inhibin secretion (P < 0.001) to pre-infusion concentrations by 36 h after sponge withdrawal. In contrast to the ovarian steroids, there was no difference between sub-groups 4a and 4b in inhibin concentrations throughout the artificial follicular phase. In group 4, replacement of FSH with LH resulted in the maintenance of immunoreactive inhibin secretion so that inhibin concentrations were higher (P < 0.05) in group 4 than in group 3 from 32 to 36 h after sponge withdrawal.
Fig. 3. Expt 1: jugular venous FSH (a) and ovarian venous oestradiol (b), androstenedione (c) and inhibin (d) concentrations in response to withdrawal of oFSH-16 (■) after 3 days with either no LH (group 3, open symbols) or pulsatile LH (group 4a non-ovulatory, shaded symbols; group 4b ovulatory, closed symbols). The insets show the secretory response to LH challenges (arrowheads) given to both groups of ewes (the scale for the y-axis is the same as for the main figure). The vertical dashed line indicates the time of progestagen sponge withdrawal and the vertical shaded bar indicates the time of the artificial LH surge. Values are means ± SEM. LH values are not shown for clarity but profiles for groups 3 and 4 were identical to those shown for groups 1 and 2, respectively, in Fig. 2.
Experiment 2

Gonadotrophin patterns. As observed in Expt 1, acute cessation of FSH infusion in group A animals resulted in a rapid decrease (P < 0.001) in peripheral FSH to basal concentrations within 8 h (Fig. 4). In group B, 'stepping down' the FSH over the initial 24 h of the artificial follicular phase resulted in a more gradual decrease in FSH so that FSH concentrations differed (P < 0.05) between groups A and B from 8 to 16 h after sponge withdrawal. Thereafter, there was no difference between the experimental groups (Fig. 4).

Similarly, there was no difference between the groups in the pattern of LH (data pooled); injections of 2.5 and 1.25 μg of LH resulted in pulses with amplitudes of 2.9 ± 0.5 and 1.2 ± 0.4 μg l−1, respectively, and the 180 mU h−1 infusion resulted in a preovulatory surge of 111 ± 20 μg l−1 (n = 18).

Follicular and endocrine responses. Three days of FSH infusion resulted in the development of 2.6 ± 0.3 and 2.8 ± 0.1 large antral follicles of diameters 3.9 ± 0.1 and 3.8 ± 0.1 mm in groups A and B, respectively (n = 9). The number and diameter of large antral follicles did not change during the artificial follicular phase in group A (2.1 ± 0.2 follicles of 4.4 ± 0.2 mm diameter on day 2; n = 9) but follicle diameter did increase in animals in group B (2.3 ± 0.2 follicles, P < 0.05; and 4.9 ± 0.1 mm diameter on day 2, P < 0.01; n = 9). Ultrasonography 6 days after the LH surge revealed that five of nine animals in group A and all nine animals in Group B had formed corpora lutea (P < 0.05) but there was no difference in ovulation rates (1.7 ± 0.2 versus 1.8 ± 0.3 in groups A (n = 5) and B (n = 9), respectively) or progesterone concentrations (7.0 ± 2.3 versus 7.2 ± 2.5 nmol l−1 in groups A (n = 5) and B (n = 9), respectively) in ovulating animals between the treatment groups.

Comparison of oestradiol profiles in ovulating animals from groups A and B showed that, while there was no difference between groups in basal or pulsatile oestradiol secretion at the time of sponge withdrawal, oestradiol concentrations differed (P < 0.01) during the initial 48 h of the artificial follicular phase (Fig. 4). In animals in which FSH was withdrawn acutely at sponge withdrawal (group A), oestradiol concentrations decreased (P < 0.05) during the initial 12 h and then increased from 12 to 24 h of the follicular phase. Thereafter, oestradiol concentrations remained stable until the expected decrease induced by the artificial preovulatory LH surge at 60 h after sponge withdrawal. In contrast, in animals in which FSH was withdrawn gradually (group B), oestradiol concentrations increased two- to threefold (P < 0.05) during the first 24 h of the follicular phase and then decreased gradually over the following 48 h, so that oestradiol concentrations did not differ between the two groups at the time of the artificial preovulatory LH surge (Fig. 4). Intensive blood sampling at 24 and 48 h after sponge withdrawal revealed that the difference in oestradiol secretion between groups A and B was due mainly to an increase in oestradiol pulse amplitude (Fig. 4 inserts).

Discussion

The present study used the GnRH-antagonist suppression-ovarian autotransplant model to examine the relative roles of the temporal changes in FSH and LH during the follicular phase on the processes of follicle development and selection. The results of these studies support the central role of FSH in follicle development but also indicate that LH plays a central role in the mechanism of follicle selection in mono-ovulatory species. Furthermore, this study provides important insights into the endocrine and ovarian consequences of different patterns of LH and FSH stimulation which have practical implications for the development of gonadotrophin stimulatory regimens designed to induce multiple or single ovulation and to maximize fertility.

In Expt 1, maintenance of FSH at above-threshold concentrations (> 0.5 ng ml−1) throughout the follicular phase resulted in multiple follicle development and multiple ovulation with pulsatile LH having little effect on follicle development but a marked effect on ovarian steroidogenesis. Withdrawal of FSH in the absence of pulsatile LH resulted in the rapid onset of atresia in the ovulatory follicle population and anovulation whereas, when FSH was withdrawn in the presence of pulsatile LH, preovulatory follicle development was maintained in half the animals, and these ewes had normal ovulation rates. Expt 2 confirmed this observation with a larger number of animals and also showed that a more gradual decrease in FSH at the start of the follicular phase resulted in normal ovulation in all ewes. Overall, these results indicate that, during a normal follicular phase, ovulatory follicles transfer their gonadotrophic dependence from FSH to LH, and that the ability of a follicle to respond to this switch in gonadotrophic support is central to the mechanism of follicle selection.

The finding that maintenance of circulating FSH at physiological concentrations supported multiple follicle development, whereas FSH withdrawal resulted in acute atresia of the large follicle population in the absence of LH, provides strong support for the theory that it is the depression in FSH that occurs normally during the follicular phase that regulates follicle selection (Baird 1983). Fundamental to this hypothesis is the concept of a threshold concentration of FSH above which there is sufficient FSH to initiate and support the development of most large antral follicles. The observation that the maintenance of FSH alone at above-threshold concentrations can stimulate the development of multiple follicles of sufficient size and maturity to ovulate is in agreement with numerous other observations in sheep (McNeilly et al., 1991) humans (Howles et al., 1994; Zelinski-Wooten et al., 1995) and cattle (Gong et al., 1996). However, the observation that acute depression of FSH to below this threshold concentration leads to immediate onset of atresia of the large follicle population, a rapid decrease in ovarian hormone secretion and a decrease in the number of follicles provides unequivocal evidence that these follicles are critically dependent on FSH in ruminants. Furthermore, these results indicate that the follicular atresia (McNeilly 1984; Henderson et al., 1986) and the decrease in ovarian hormone secretion that result from treatment of ewes with steroid-free follicular fluid (Baird et al., 1990; Campbell et al., 1991a) or inhibit (Campbell et al., 1996) are due to depression of FSH to below threshold concentrations. Overall, these data provide strong support for the FSH 'threshold' hypothesis. The fact that the
switch from below to above threshold was represented by small (about 0.4 ng ml\(^{-1}\)) changes in jugular venous FSH concentrations may explain the difficulty experienced by many investigators in relating FSH concentrations to ovulatory potential (for review, see Scaramuzzi and Campbell, 1990).

The finding that low amplitude–high frequency LH pulses, while having major effects on ovarian steroid secretion, had no effect on the number of ovulatory follicles, ovulation rate and subsequent luteal function indicate that LH is not required in situations in which FSH is above ‘threshold’ concentrations. Although these data support some previous observations (Campbell et al., 1998), they contrast with other data reporting suppressive effects of LH on superovulatory responses to FSH in sheep (Wright et al., 1981, Chupin et al., 1987), cattle (Murphy et al., 1984) and rats (Armstrong et al., 1989), and the stimulatory action of FSH on follicular growth in GnRH-agonist suppressed animals (Picton et al., 1990b). The most likely explanation for this discrepancy is the dose of LH used. In the present experiment, the doses of LH were chosen and confirmed to induce pulses of similar magnitude and frequency to those observed during a normal follicular phase (Campbell et al., 1990) while greater doses of LH were used in other reports. This explanation is supported by data from cultured ruminant follicular cells showing that high doses of LH inhibit steroidogenesis (Berndtson et al., 1995; Campbell et al., 1998b).

The removal of FSH support at the time of ‘luteolysis’ or
sponge withdrawal in combination with high frequency–low amplitude LH pulses was the treatment designed to mimic most closely the endocrine changes observed during the transition from the luteal to the follicular phase and, thus, replicate normal follicle selection. The results indicate that this treatment placed severe selection pressure on the large follicle population with about half the ewes treated in this way (groups 4 and A of Expts 1 and 2, respectively) failing to ovulate. However, those that did ovulate did so at a rate equivalent to the normal ovulation rate of this breed. The most likely explanation for the sub-optimal response to this stimulatory regimen is that it did not precisely mimic the normal luteal to follicular phase transition. Comparison of hormonal profiles between a normal cycle and the current treatment regimen indicate that the time taken for FSH to decrease to below 'threshold' values after 'luteal regression' was shorter by about 12 h for the induced regimen compared with a natural follicular phase (about 24 h). Thus, it was hypothesized that a more gradual decrease in FSH would allow the large antral follicles more time to transfer from FSH to LH dependence. This hypothesis was tested in Expt 2, which incorporated a step-down in FSH in conjunction with 3,2,1 pulsatile LH from the time of luteal regression, and the results show that this regimen resulted in all animals ovulating the normal number of follicles. Therefore, these results indicate that ovulatory-sized FSH-dependent follicles can transfer their gonadotrophic dependence from FSH to LH, if the LH is delivered as a series of high frequency–low amplitude pulses. Furthermore, it is hypothesized that it is the ability of a follicle to respond to this switch in gonadotrophic support, presumably through the acquisition of a sufficient number of LH receptors on the granulosa cells, that is the central mechanism of follicle selection. This hypothesis is supported by the observation that large antral follicles become LH-dependent when exposed to high frequency–low amplitude LH pulses (Dobson et al., 1997b).

The results of the present study confirm the previous findings with this model system (Campbell et al., 1998) that GnRH-antagonist treatment inhibits both immunoactive and bioactive LH secretion and that FSH-stimulated large ovulatory follicles secrete very little androstenedione or oestradiol in the absence of LH drive. Furthermore, the replacement of LH pulses restores ovarian steroid secretion, thus emphasizing the fact that the amount of ovarian steroid secretion depends not only on the presence of steroidogenic follicles in the ovary but also on the degree of LH drive. Basal steroid secretion in animals in group 1 did increase over the 'follicular phase', albeit at a rate 10–20 times lower than in group 2 animals. It is possible that this amount of steroid secretion is constitutive, but it is equally likely that it is stimulated by endogenous LH or by LH contamination in the FSH preparation. In contrast to the steroids, ovarian immunoactive-inhibin secretion accurately reflected the large antral follicle population, confirming that inhibin secretion is not acutely responsive to LH (Campbell et al., 1989) and that large antral follicles are a major source of ovarian inhibin secretion (Campbell et al., 1991b; Mann et al., 1992; Engelhardt et al., 1993).

Gonadotrophin regimens designed to stimulate follicular development are widely used in animal production and clinical medicine. The results of the present study provide important insights into the consequences of different patterns of LH and FSH stimulation. It is clear that stimulatory regimens that use FSH alone in GnRH-antagonist suppressed individuals are unphysiological in terms of the low ovarian oestradiol and androgen secretion. Ovarian steroids are thought to have key autocrine and paracrine actions during follicular (Hillier, 1987) and oocyte (Moor et al., 1980) development, in addition to their major endocrine actions in the control of pituitary gonadotrophin release (Karsch et al., 1984), the responsiveness of the uterus to luteolytic signals (Lamming and Mann, 1995), gamete and zygote transport (Harper, 1988) and the induction of behavioural oestrus (Baird and McNeilly 1981; FabreNys et al., 1993). Therefore, stimulatory regimens that result in a hyposteroidal environment may result in sub-optimal fertility, and this may become an increasing problem with the adoption of GnRH antagonists in preference to GnRH agonists (Karnitis et al., 1994; Zeleinski-Wooten et al., 1995) and the use of highly purified FSH preparations in preference to those with both LH and FSH activity in human fertility treatment (Howles et al., 1994). Conversely, although these results also show that normal patterns of ovarian steroid secretion can be attained when FSH and pulsatile LH are combined, the amount of ovarian steroid secretion stimulated was three- to fourfold higher than normal (Campbell et al., 1994). Therefore, this stimulatory regimen is directly analogous to superovulatory regimens that include both FSH and LH activity, such as equine chorionic gonadotrophin (Murray et al., 1994) or human menopausal gonadotrophin (Soderstromanttila et al., 1996; Fauser and Heusden, 1997). Given the key endocrine roles of the steroids detailed above, it is possible that many of the deleterious effects of hyperstimulation on oocyte quality, sperm and oocyte transport and early embryo development (Hawk 1988; Armstrong 1993; Dawood 1996) can be attributed to the hypersteroidal endocrine environment resulting from these types of stimulatory regimen. Therefore, to attain a normal steroidal environment, it is likely that regimens would have to be developed that incorporate pure FSH in combination with sub-physiological doses of LH.

In conclusion, the results of the present study show that both the decrease in peripheral FSH and the increase in LH pulse frequency that occur during the follicular phase are important to the process of follicle selection, in that FSH-dependent follicles can withstand withdrawal of FSH by transferring their gonadotrophic requirement from FSH to LH if LH is delivered as a series of high frequency, low amplitude pulses. These data provide direct support for the hypothesis that the transfer of gonadotrophic dependence from FSH to LH is central to the mechanism of follicle selection.

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