Corpora lutea induced by gonadotrophin-releasing hormone treatment of anoestrous Welsh Mountain ewes: reduced sensitivity to luteinizing hormone *in vivo* and to chorionic gonadotrophin *in vitro*

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Abstract

Seasonally anoestrous Welsh Mountain ewes received 250 ng gonadotrophin-releasing hormone (GnRH) every 2 h, with (Group 1; $n = 13$) or without (Group 2; $n = 14$) progesterone priming for 48 h. Fourteen control ewes (Group 3) were studied during the luteal phase in the breeding season. Animals in Group 4 ($n = 12$) received progesterone priming followed by 250 ng GnRH at increasing frequency for 72 h, while ewes in Group 5 ($n = 13$) were given three bolus injections of 30 μ g GnRH at 90-min intervals. All treatment regimens induced ovulation. However, only corpora lutea (CL) from ewes in Group 3 (breeding season) or Group 4 exhibited normal luteal function. Luteal luteinizing hormone (LH) receptor levels were significantly higher on day 12 than day 4, and CL from groups with adequate CL (3 and 4) had significantly higher ¹²⁵I-human chorionic gonadotrophin (hCG)-binding levels than the three groups with inadequate CL on day 12. LH-binding affinity was unchanged. Exogenous ovine LH (10 μ g) *in vivo* on days 3 or 11 after ovulation induced a pulse of progesterone in ewes with adequate CL; however, ewes in Groups 1, 2 and 5 showed no significant response. Basal progesterone secretion *in vitro* was significantly greater on day 4 than on day 12. Maximal steroidogenic responses of adequate and inadequate CL to hCG and to dibutyryl cyclic-3',5'-AMP were similar at both stages of the luteal phase. However, the EC₅₀ for hCG on days 4 and 12 was 10-fold lower for groups with an adequate CL (0.1 IU hCG/ml) than for inadequate-CL groups (1 IU hCG/ml; $P < 0.05$). Thus, in addition to the well-characterized premature sensitivity of GnRH-induced inadequate CL to endometrial luteolysis, we have shown (1) a marked decrease in total number of cells in the CL, a profound reduction in vascular surface area, and a decrease in mean large luteal cell volume (with no change in large luteal cell numbers), (2) decreased luteal LH receptor and progesterone content compared with adequate CL and (3) that CL that were becoming, or were destined to become, inadequate failed to respond to ovine LH *in vivo* and were 10-fold less sensitive to hCG in terms of luteal progesterone secretion *in vitro*.

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Introduction

Inadequate luteal function contributes significantly to infertility (Hunter 1991, Garverick *et al.* 1992). Transient inadequate luteal function occurs naturally in sheep, for example, in early postpartum (Wright *et al.* 1983, 1984, Braden *et al.* 1989), at puberty and during the transition from anoestrus to the breeding season (Legan *et al.* 1985), and following ram-induced ovulation during anoestrus (Martin *et al.* 1986). Induction of ovulation in seasonally anoestrous ewes by the administration of luteinizing hormone (LH) or gonadotrophin-releasing hormone (GnRH;

Crighton *et al.* 1973) or GnRH agonist (McNeilly *et al.* 1981) generates inadequate corpora lutea (CL), and such CL have been widely used as a model to study naturally occurring inadequate luteal function. The CL formed tend to be smaller and secrete less progesterone *in vivo* and *in vitro* than CL of the breeding season (McNeilly & Land 1979, McNeilly *et al.* 1981). Increasing the frequency of LH (McNeilly *et al.* 1982) or GnRH pulses (Crighton *et al.* 1975, McLeod *et al.* 1982a, 1982b, Southee *et al.* 1988a, 1988b) to mimic the final stages of follicular maturation during the breeding season increased ovulation rate during seasonal anoestrus but still gave rise frequently to

abnormal CL. However, the incidence of adequate luteal phases during anoestrus was increased significantly by progesterone pretreatment prior to induction of ovulation with GnRH (McLeod *et al.* 1982*b*, Legan *et al.* 1985). Progesterone prolonged the period between GnRH injection and the onset of the LH surge (McLeod and Haresign 1984, Southee *et al.* 1988*a*), lengthening the final phase of follicular maturation, but had no effect on the onset, amplitude or duration of the LH surge (Wright *et al.* 1983, Legan *et al.* 1985).

A number of different mechanisms have been suggested to be responsible for the formation of inadequate luteal phases in sheep, including defective maturation of the pre-ovulatory follicle (Keisler & Keisler 1989, Hunter *et al.* 1986, White *et al.* 1987, Southee *et al.* 1988*a*, Khalid *et al.* 1997, Lund *et al.* 1999, Bartlewski *et al.* 2001), perhaps due to an inappropriate pattern of LH stimulation of the follicle prior to the LH surge (McLeod *et al.* 1982*a*, Wright *et al.* 1983, 1984), attenuation of the LH surge (Bartlewski *et al.* 2004), inadequate luteinization (Atkinson 1988, McNeilly *et al.* 1981, Hunter *et al.* 1988), inappropriate luteal support of the new CL (Hunter *et al.* 1988) and/or increased susceptibility to luteolytic stimuli (Hunter *et al.* 1989, Beard & Hunter 1996) arising from the uterus (Southee *et al.* 1988*b*, Hu *et al.* 1991, though see Rahmanian and Murdoch 1987).

In order to distinguish factors that influence luteal function from other seasonal factors, we administered GnRH regimens that were predicted to give rise to adequate or inadequate CL to Welsh Mountain ewes during seasonal anoestrus, and assessed the incidence of ovulation, luteal morphology and hormonal responsiveness of the CL formed *in vivo* and *in vitro* at two stages of development.

Materials and Methods

Materials

All fine chemicals and reagents were purchased from Aldrich (Gillingham, Dorset, UK) or BDH (Poole, Dorset, UK). Radiolabelled [1,2,6,7-³H]progesterone (100 Ci/mmol) and Na¹²⁵I (100 mCi/ml) were purchased from Amersham International. ¹²⁵I-Labelled pregn-4-ene-3,20-dione was purchased from Sigma Chemical Co. Highly purified human chorionic gonadotrophin (hCG; Profasi; 15 000 IU/vial) for radio-iodination was purchased from Serono (Welwyn Garden City, Herts, UK). hCG for the measurement of non-specific binding (Chorulon; 5000 U/vial) was from Intervet Laboratories (Cambridge, UK). Antibodies for the assay of progesterone (S-361), prolactin (R2532), follicle-stimulating hormone (FSH) (M94) and LH (R29) were the generous gifts from Professor Alan McNeilly (MRC HRSU, Edinburgh, Scotland, UK). Normal rabbit serum and donkey anti-rabbit serum were purchased from the Scottish Antibody Production Unit, Carlisle, Scotland, UK.

In vivo studies

Sixty-seven Welsh Mountain ewes of proven reproductive history were kept unmated over the preceding breeding season and maintained outdoors under natural lighting conditions at Dryden Field Laboratory, Roslin, Midlothian, Scotland. Ewes were weighed, ranked for condition and allocated randomly to the appropriate groups. The study was performed in three parts, as follows.

First, during seasonal anoestrus (June) 13 ewes (Group 1) received 250 ng GnRH (Ayerst-Wyeth Pharmaceuticals, Maidenhead, Berks, UK) every 2 h for 48 h following a 12-day treatment with progesterone (three progesterone implants containing 375 mg progesterone in a silicone elastomer matrix; Abbott Laboratories). Implants were placed in the axillary region and removed immediately after the second GnRH injection. Plasma progesterone concentrations at the end of the 12-day treatment were 3.9 ± 0.4 ng/ml (mean $\pm$ S.E.M.). Group 2 ewes ($n = 14$) received the same regimen of GnRH treatment as Group 1, but without progesterone pretreatment (McLeod *et al.* 1982*b*). Animals were slaughtered on either day 4 or day 12 post-ovulation.

Secondly, the following March (late breeding season), the oestrous cycles of 14 ewes (Group 3) were synchronized (Chronogest progestagen pessaries; Intervet Laboratories). Ewes were left untreated for the first cycle. Following detection of oestrus of the second cycle with a vasectomized ram, blood samples were taken twice daily from the jugular vein until slaughter on day 4 or day 12 (oestrus = day 0).

Finally, in mid-June (seasonal anoestrus), 26 ewes were weighed and allocated randomly to two groups. Twelve ewes (Group 4) were progestagen-treated for 7 days (Chronogest progestagen pessaries) and then received injections of 250 ng GnRH for 72 h at increasing frequencies (every 3 h for 24 h, every 2 h for 24 h and finally every hour for a further 24 h). Thirteen ewes (Group 5) received no progestagen treatment, but were given three bolus injections of 30 µg GnRH at 90-min intervals.

Blood sampling

Groups 1, 2, 4 and 5 were sampled every 15 min for 12 h before treatment started. Groups 1 and 2 were sampled every 2 h throughout the 48 h of GnRH injection, and every 4 h for a further 48 h. In addition, 15-min samples were collected for 6 h after the start of the 2-h GnRH injections. Ewes were then sampled twice daily until slaughter. Group 3 ewes were sampled twice daily until slaughter. Group 4 ewes were sampled before each injection of GnRH (i.e. with increasing frequency for 72 h), every 4 h for a subsequent 24 h, and then twice daily until slaughter. In addition, there was also a period of intense (15-min) sampling from the start of the 2 h injections. All blood samples at intervals of 4 h or less were taken via an indwelling venous catheter (3 ml samples). Twice-daily samples (7 ml) were collected by jugular venepuncture.

Responsiveness to LH *in vivo*

To test the response to a physiological dose of ovine LH (oLH) *in vivo*, ewes were sampled for a control period, then given a bolus intravenous injection of oLH (10 µg NIH-LH-S15) on day 3 or day 11. Blood samples were collected at 15 min intervals for a further 4 h.

In vitro studies

Tissue processing

Ovaries were collected from ewes at slaughter on days 4 or 11 of the luteal phase, and transported to the laboratory on ice within an hour. CL were excised, trimmed free of fat and connective tissue, weighed, and divided into roughly equal portions for tissue incubations, receptor assays and morphological studies.

Histological studies

Wedge-shaped segments of luteal tissue (representing 20–25% of each CL) were taken from five or six animals from each group (from ewes that had shown no premature fall in serum progesterone) and fixed by immersion in 2% glutaraldehyde/0.1 M cacodylate buffer, pH 7.4, for 2 h, then in 5% glutaraldehyde/0.1 M cacodylate buffer for a further 2 h. Fixed tissue was sliced into 1 mm³ pieces with new razor blades, then 5–10 pieces were randomly selected and post-fixed in osmium tetroxide (2%) for 2 h, dehydrated in alcohol and embedded in epon araldite. Semi-thin sections (1 µm) were cut and stained with Toluidene Blue for morphometric analysis. Cell number and cell size were measured in five squares of a micrometer grid for four random separate fields of view for each section by defocusing the microscope and moving the stage along a predetermined track. For each CL, a total of 100–200 cells was counted.

To estimate the surface area of vasculature per unit volume of tissue, the number of intercepts of the surface and a bar on an M42 (21-bar) grid (L4062 eyepiece graticule; Agar Aids, Agar Scientific, Stanstead, UK) were counted at random by defocusing the microscope and moving the stage along a predetermined track. Vascular surface area was calculated from the formula $S_v = 2 \times I/L$ where I is the number of intercepts and L is the total length of the grid bars, taking into account the magnification (Weibel 1989, Cruz-Orive & Weibel 1990, Lecocq 1993).

Using a Tektronix (Thurlby Thandar Instruments, Huntingdon, Cambs, UK) image analyser, all nucleated cells with a diameter of >15 µm within a square of known size and section of known thickness were measured. Cells were only counted if their nucleus fell within or transected the upper and/or left side of the grid square. Perimeters of cells were traced and measured, areas of profiles estimated and mean cell volume calculated. The number of cells per unit volume of fixed tissue (N_v) was estimated from the sum of all blocks using the formula $N_v = 1/\beta N_a^{1.5}/V_v^{0.5}$ where N_a is the number of

cells measured, V_v is the volume fraction of the cells (i.e. the combined area of all measured cells divided by the true area of the section studied) and β is a correction factor based on nuclear shape ($\beta = 1.382$ for luteal cells; Rodgers *et al.* 1984). The number of cells per CL was the product of this N_v and the volume of the CL. Mean cell volume was calculated from the mean cell diameter of two measurements at right angles, assuming that luteal cells are spherical ($4/3\pi r^3$).

Progesterone secretion *in vitro*

Duplicate aliquots of minced tissue from each CL were incubated at 37°C in either 1 ml M199 alone (Flow Laboratories, Irvine, Scotland) or M199 supplemented with hCG (Chorulon; 10⁻⁴ to 10² IU/ml in 10-fold increments) or *N*⁶,2'-*O*-dibutyl cyclic-3',5'-AMP (dbcAMP; 0.0625–0.5 mM), as described in the figure legends. After incubation, tubes were centrifuged (5000 g for 10 min) and tissue pellets and media stored separately at -20°C. Tissue pellets were homogenized and assayed for protein (Lowry *et al.* 1951) to correct steroid secretion for differing amounts of tissue. Incubation media were assayed for progesterone by RIA (Scaramuzzi & Baird 1977).

Assays

oLH was measured by RIA using the method of McNeilly *et al.* (1985). Assay sensitivity was 0.2 ng/ml, and intra- and inter-assay coefficients of variation were 5.2 and 12.1% respectively. Plasma FSH concentrations were measured using the RIA described by McNeilly *et al.* (1976). Assay sensitivity was 4 ng/ml, and intra- and inter-assay coefficients of variation were 4.9 and 11.8% respectively. Prolactin was measured using the assay of McNeilly & Andrews (1974). Assay sensitivity was 0.1 ng/ml, and intra- and inter-assay coefficients of variation were 8.1 and 11.2% respectively.

Serum progesterone concentrations were measured in duplicate aliquots (200 µl) of plasma samples following extraction with 2 ml freshly distilled peroxide-free petroleum ether. After vortexing for 30 min, samples were frozen in a dry ice/methanol bath, and the organic phase dried under nitrogen. Extraction efficiency was estimated by recovery of [³H]progesterone (1000 c.p.m.). Dried samples were reconstituted in 300 µl 0.1% gelatin/PBS, diluted and radioimmunoassayed (Djahanbakhch *et al.* 1981) using ¹²⁵I-labelled pregn-4-ene-3,20-dione tracer. Assay sensitivity was 0.1 ng/ml, and intra- and inter-assay coefficients of variation were 7.2 and 10.9% respectively for five separate assays. Culture medium did not interfere with the assay, so progesterone content of media was measured without solvent extraction.

Assay of occupied and unoccupied LH receptors

Unoccupied LH receptors were measured by specific binding of ¹²⁵I-labelled hCG (Profasi; radio-iodinated to

a specific binding activity of 35 Ci/g estimated by self-displacement assay) to aliquots (10–100 μ l) of ovine luteal homogenates as described previously (Bramley *et al.* 1987). Values of $B_{\max}$ and K_a for luteal LH/hCG receptors were estimated from Scatchard plots constructed from triplicate measurements of specific binding at 8–10 concentrations (0.5–30 pM) of 125 I-hCG (Bramley *et al.* 1987).

Preliminary experiments demonstrated that bound 125 I-hCG was released with high efficiency following incubation on ice at pH 3.0 (Fig. 1A) for 5 min (Fig. 1B). Therefore occupied LH receptor levels were measured by dissociation of bound oLH from aliquots (100 μ l) of luteal homogenate with 100 μ l ice-cold 0.1 M citrate buffer, pH 3.0. After 5 min, pH was immediately restored to 7.4 by addition of 175 μ l 1 M Tris-HCl, pH 7.5, tubes were centrifuged at 30 000 *g* for 15 min, and the oLH released

was measured in aliquots of the supernatant by immunoassay. Receptor concentrations were adjusted for DNA content (Burton 1956) using calf thymus DNA as standard.

Analysis of data

Onset of the preovulatory LH surge was considered to have occurred when plasma LH levels exceeded 15 ng/ml, provided that levels in two consecutive samples were greater than the mean of the previous two samples, and at least one of the peak samples exceeded the mean basal level by more than three times the coefficient of variation of the assay (Backstrom *et al.* 1982).

Luteal function was defined as adequate when plasma progesterone concentrations (1) rose within 4 days of the LH surge, (2) remained elevated for at least 8 days and (3) reached concentrations of >1.5 ng/ml for at least 2 consecutive days (Hunter *et al.* 1986).

Statistics

The incidence of ovulation and adequacy of luteal function were compared between groups using the χ^2 test or Fisher's exact test. Hormone profiles were compared using two-way analysis of variance with repeated measures, followed by Duncan's multiple-range test where appropriate. Morphometric parameters were compared between groups using Student's *t* test.

Results

Ovulation and luteal function

The proportion of ewes in each treatment group that ovulated in response to GnRH treatment ranged from 5/13 to 9/13 (Table 1). The remaining animals showed no elevation in plasma progesterone, and no evidence of luteal structures at dissection. All ovulating ewes had only a single CL: there were no multiple ovulations. There were no statistically significant differences in the proportion of ewes ovulating between the groups. However, only ewes in Group 3 (breeding season; $n = 13/13$) and Group 4 (progesterone pretreated with increasing GnRH frequency for 72 h; $n = 10/12$) showed adequate luteal function. The interval between the last bolus GnRH injection and the onset of LH surge in Group 5 was only 1.5 h, and the LH surge was significantly reduced and foreshortened (Table 1). However, there were no significant differences in duration or magnitude of the LH surge between Groups 1 and 4, although time to the onset of the LH surge was longer for Groups 3 and 4 compared with Group 2 ($P < 0.02$), but not Group 1.

Plasma gonadotrophin concentrations

There were no significant differences in basal LH concentrations (1.3 ± 0.2 ng/ml), LH pulse frequency (1.7 ± 0.5 pulses/12 h) or LH pulse amplitude (10.7 ± 2.8 ng/ml)

Figure 1 Dissociation of bound 125 I-hCG from ovine luteal tissue. Triplicate aliquots (500 μ l) of mid-luteal ovine CL were incubated with 500 000 c.p.m. 125 I-hCG tracer (specific activity of binding, 35 Ci/g) in 0.1% BSA/Tris-HCl buffer, pH 7.4, overnight at 20 °C. Bound hormone was recovered by centrifugation at 15 000 *g* for 15 min and the pellet resuspended in BSA/Tris buffer. Triplicate aliquots (100 μ l) were then incubated in ice-cold 0.1 M acetic acid/sodium citrate buffer of the appropriate pH for 5 min (A), or at pH 3.0 for the appropriate time (B). Tubes were then centrifuged at 30 000 *g* for 30 min, and the pellet (○) or supernatant (●) counted for 125 I radioactivity. Points are means $\pm$ S.E.M. from a single representative experiment in triplicate. Similar data were obtained in four less-complete experiments.

Table 1 Pituitary and ovarian responses to GnRH injection. Ovulation was assessed by a rise in plasma progesterone concentrations and the presence of a CL or corpus albicans at slaughter. Data are means $\pm$ S.E.M.

Group	Treatment	Proportion ovulating	Duration of LH surge (h)	LH surge (ng/ml)	% Adequate luteal phases	Interval between GnRH injection and LH surge (h)
1	250 ng GnRH every 2 h for 48 h Progesterone pretreated	5/13	16.6 $\pm$ 2.3	151 $\pm$ 17	0	37.2 $\pm$ 3.0
2	250 ng GnRH every 2 h for 48 h No progesterone pretreatment	9/14	14.1 $\pm$ 3.7	144 $\pm$ 19	0	22.0 $\pm$ 8.5
3	Breeding season	13/13	16.4 $\pm$ 2.9	137 $\pm$ 20	100	50.0 $\pm$ 7.7 ^b
4	250 ng for 72 h at increasing frequency Progesterone pretreated	10/12	18.3 $\pm$ 4.2	176 $\pm$ 23	100	52.0 $\pm$ 8.9 ^b
5	30 μ g GnRH $\times$ 3 No progesterone pretreatment	9/13	7.8 $\pm$ 2.1 ^a	95 $\pm$ 9 ^a	0	(1.5)

^aSignificantly different from other groups ($P < 0.05$).

^bSignificantly different from Group 2 ($P < 0.02$).

between groups before the start of treatment, and no significant differences in LH pulse amplitude in response to 2 h GnRH injections for Groups 1, 2 and 4 (data not shown). Similarly, mean FSH levels before the start of treatment (58 ± 7 ng/ml) and FSH response to GnRH (mean concentration, 83 ± 9 ng/ml) were similar in all groups ($P > 0.05$). There were no significant differences between treatment groups in prolactin concentrations (118 ± 27 ng/ml) throughout the luteal phase. However, irrespective of treatment group, mean prolactin concentrations were significantly lower ($P < 0.02$) in ewes with short-lived luteal phases (112 ± 8 ng/ml) compared with ewes in which CL survived until day 12 (134 ± 4 ng/ml; $P < 0.02$).

Plasma progesterone concentrations

Plasma progesterone concentrations of ewes that ovulated and were tracked up to day 12 are shown in Fig. 2A. Mean progesterone concentrations for treatment Groups 1, 2 and 5 showed no significant differences (until beyond day 10, when mean progesterone levels in Group 5 ewes fell).

Response to LH stimulation in vivo

Injection of oLH (10 μ g) generated a similar pulse of plasma LH for ewes in all treatment groups (mean pulse amplitude, 3.3 ± 0.7 ng/ml). During the breeding season (Group 3), the LH pulse was followed by a significant elevation in plasma progesterone concentrations on both day 3 (Fig. 3A) and day 11 (Fig. 3D). Similarly, ewes in Group 4 also showed a sharp rise in progesterone (Fig. 3B and E). However, there was no significant plasma progesterone increase in response to LH injection in ewes from Groups 1, 2 and 5 on either day 3 (Fig. 3C) or (in the four ewes in which a CL was still present) on day 11 (Fig. 3F).

Some ewes with adequate (Fig. 4A and B) or inadequate CL (Fig. 4C and D) exhibited an endogenous LH pulse during the course of the 4 h sampling period. In ewes with adequate CL, there was a significant increase in plasma progesterone in response to both an exogenous oLH and endogenous LH pulse on both day 3 (Fig. 4A)

Figure 2 Luteal plasma progesterone levels in ewes from the different treatment groups. (A) Anoestrous ewes or ewes during the breeding season were treated as described in the Materials and Methods section and blood samples collected twice daily for 12 days. Plasma progesterone levels were measured by immunoassay. Symbols used: Group 1 (Δ ; $n = 5$); Group 2 ($\blacktriangle$; $n = 9$); Group 3 ($\bullet$; $n = 13$); Group 4 ($\circ$; $n = 10$); Group 5 ($\square$; $n = 9$). Points are means for all ewes in each treatment group. (B) Ewes during the breeding season (Group 3; $\blacksquare$; $n = 13$) or anoestrous ewes (from Groups 1 and 2) having a transient ($\circ$; $n = 4$) or a persistent CL ($\bullet$; $n = 4$) as assessed retrospectively. Points are means $\pm$ S.E.M.

Figure 3 Plasma progesterone concentrations in response to oLH injection *in vivo*. Anoestrous ewes or breeding-season ewes were treated as described in the Materials and Methods section and injected with 10 µg oLH (arrowhead) on either day 3 (A, C, E) or day 11 (B, D, F) of the luteal phase. Blood samples were collected every 15 min for 4 h, and plasma levels of oLH (○) and progesterone (●) were measured by immunoassay. (A) Day 3, Group 3 (breeding season; $n = 6$); (B) day 3, Group 4 (adequate CL; $n = 5$); (C) day 3, Groups 1, 2 and 5 (inadequate CL; $n = 10$); (D) day 11, Group 3 (breeding season; $n = 7$); (E) day 11, Group 4 (adequate CL; $n = 5$); (F) day 11, Groups 1, 2 and 5 (inadequate CL; $n = 13$). Points are means for 4–8 ewes per group.

and day 11 (Fig. 4B). In contrast, ewes in the inadequate-treatment groups (1, 2 and 5) failed to respond to exogenous or endogenous oLH at either stage of the luteal phase (Fig. 4C and D).

Characteristics of CL

All groups showed a significant increase in luteal weight between days 4 and 12 (Table 2). CL from the breeding season were significantly heavier on days 4 and 12 than those induced by ovulation during anoestrus. Luteal progesterone content was significantly higher in breeding-season CL compared with the three inadequate CL groups. Intermediate luteal progesterone levels were observed in CL from Group 4 (adequate induced) ewes.

Unoccupied luteal LH receptor levels rose significantly between days 4 and 12 in all groups and were significantly higher in the two groups with adequate CL (Groups 3 and 4) on day 12. Occupancy of LH receptors ranged between 3 and 13%, and increased significantly between day 4 and day 12 in Groups 3 and 4. However, there was no significant difference in LH receptor affinity between ewes with adequate or inadequate groups CL on day 12 (Group 3, $K_a = 0.48 \pm 0.12$ ($n = 7$); Group 4, $K_a = 0.38 \pm 0.1$ ($n = 5$); Groups 1, 2 and 5 combined, $K_a = 0.4 \pm 0.12$ ($n = 12$); all K_a data are $\times 10^{10} \text{ M}^{-1}$; data not shown).

Morphological studies

There were no significant differences between the different morphological characteristics of CL from treatment Groups 1, 2 and 5 in terms of luteal weight, hCG receptors, progesterone content, basal or maximal progesterone secretion *in vitro*, or luteal sensitivity (EC_{50}) to hCG (see below). Therefore data from CL from Groups 1, 2 and 5 were combined for comparison of their morphology with groups with adequate CL (Group 3 and 4).

In all treatment groups there was a significant increase in the total number of cells in CL recovered on day 12 compared with day 4 (Table 3). Moreover, total cell number per CL was significantly greater in CL of the breeding season than either adequate (Group 4) or inadequate (Groups 1, 2 and 5) GnRH-induced CL. In contrast, the number of large luteal cells (diameter $> 15 \mu\text{m}$) was similar at both stages of the luteal phase, and between treatment groups (Table 3). However, luteal cell volume was significantly greater in breeding season CL (day 12 vs day 4; $P < 0.01$), and in Group 3 ewes versus inadequate groups ($P < 0.05$) on day 12. Luteal vascular surface area was significantly greater on day 12 than on day 4 in all groups, and in breeding season CL compared with adequate (Group 4) or inadequate (Groups 1, 2 and 5) GnRH-induced CL ($P < 0.05$; Table 3).

Figure 4 Plasma progesterone concentrations in response to exogenous and endogenous oLH pulses *in vivo*. Anoestrous ewes were treated as described in the Materials and Methods section and injected with 10 µg oLH (solid arrowheads) on either day 3 (A, B) or day 11 (C, D) of the luteal phase. Blood samples were collected every 15 mins for 4 h, and plasma levels of oLH (○) and progesterone (●) were measured by immunoassay. (A) Day 3 (adequate CL; Group 3); (B) day 11 (adequate CL; Group 4); (C) day 3 (inadequate CL; Group 5); (D) day 11 (inadequate CL; Group 5). Points are means of triplicate assays for a single representative animal from each group. Solid arrowheads indicate point of exogenous oLH injection; open arrowheads indicate an endogenous LH pulse.

In vitro studies of progesterone secretion

Pilot experiments showed that progesterone secretion with 0, 0.1 or 10 IU hCG/ml increased linearly for up to 3 h (Fig. 5A). Dose–response curves for hCG became progressively flatter with increasing duration of incubation

due to rising basal secretion, although EC_{50} for hCG did not change (Fig. 5B). A standard incubation time of 2 h was therefore used for dose–response studies of hCG stimulation of progesterone secretion by luteal tissue minces from experimental groups. Basal progesterone

Table 2 Characteristics of CL induced by GnRH during anoestrus and CL formed during the breeding season. All luteal structures were included in the analysis, irrespective of premature falls in plasma progesterone levels. Data are means $\pm$ S.E.M.

Group	Day	CL number	Luteal weight (mg)	Progesterone content (pg/mg protein)	¹²⁵ I-hCG binding (pg/µg DNA)	
					Unoccupied	Occupied
1	4	2	110 $\pm$ 63	14.2 $\pm$ 2.1	3.8 $\pm$ 1.9	n.d.
2	4	4	118 $\pm$ 35	15.8 $\pm$ 3.7	3.5 $\pm$ 2.1	n.d.
3	4	6	203 $\pm$ 29 ^a	85.3 $\pm$ 3.6 ^a	5.7 $\pm$ 1.2	0.34 $\pm$ 0.04
4	4	5	113 $\pm$ 18	45.9 $\pm$ 2.8 ^a	4.7 $\pm$ 1.0	0.28 $\pm$ 0.03
5	4	4	127 $\pm$ 12	13.8 $\pm$ 1.7	2.1 $\pm$ 0.7	0.27 $\pm$ 0.05
1	12	3	340 $\pm$ 30 ^b	19.7 $\pm$ 2.3	8.7 $\pm$ 2.9 ^b	n.d.
2	12	5	399 $\pm$ 10 ^b	16.2 $\pm$ 4.1	9.2 $\pm$ 2.3 ^b	n.d.
3	12	7	569 $\pm$ 50 ^{a,b}	47.5 $\pm$ 5.2 ^{a,b}	19.5 $\pm$ 4.3 ^{a,b}	0.65 $\pm$ 0.08 ^b
4	12	5	397 $\pm$ 34 ^b	28.7 $\pm$ 3.5 ^{a,b}	14.8 $\pm$ 2.1 ^{a,b}	0.58 $\pm$ 0.07 ^b
5	12	5	338 $\pm$ 91 ^b	15.6 $\pm$ 2.8	7.9 $\pm$ 3.2 ^b	0.31 $\pm$ 0.04

n.d., not determined.

^aSignificantly different from inadequate groups ($P < 0.05$).

^bSignificantly different from same group on day 4 ($P < 0.05$).

Table 3 Morphological characteristics of breeding-season and GnRH-induced CL. All luteal structures were included in the analysis, irrespective of premature falls in plasma progesterone levels. Data are means $\pm$ S.E.M.

Group	Day	CL number	Total cell number (millions)	Luteal cell number (millions)	Luteal cell volume ($\mu\text{m}^3 \times 10^{-3}$)	DNA content (mg/CL)	Vascular surface area (cm^2)
3	4	6	87 $\pm$ 13	51 $\pm$ 10	5.9 $\pm$ 1.8	0.71 $\pm$ 0.11	61 $\pm$ 8
4	4	5	63 $\pm$ 10	45 $\pm$ 8	5.4 $\pm$ 1.3	0.52 $\pm$ 0.08	54 $\pm$ 9
1, 2, 5	4	10	56 $\pm$ 11	48 $\pm$ 6	4.9 $\pm$ 1.4	0.46 $\pm$ 0.09	48 $\pm$ 7
3	12	7	274 $\pm$ 22 ^{*a}	76 $\pm$ 9	16.9 $\pm$ 2.4 ^{*a}	2.23 $\pm$ 0.18 ^{*a}	239 $\pm$ 15 ^{*a}
4	12	5	194 $\pm$ 13 ^{*a,b}	53 $\pm$ 8	10.3 $\pm$ 2.1	1.58 $\pm$ 0.11 ^{*a,b}	172 $\pm$ 12 ^{*a,b}
1, 2, 5	12	13	135 $\pm$ 16 ^{*a,c}	49 $\pm$ 8	9.3 $\pm$ 1.9 ^b	1.10 $\pm$ 0.13 ^{*a,c}	112 $\pm$ 11 ^{*a,c}

Values within columns with different superscript letters are significantly different ($P < 0.05$).

*Significantly different from day 4 ($P < 0.01$).

secretion by luteal tissue from day 4 of the luteal phase was significantly higher ($P < 0.05$) than on day 12 of the luteal phase for all groups (cf Fig. 6A and B). However, maximal responses to hCG or dbcAMP were similar on day 4 and day 12 for all treatment groups (Fig. 6). Dose-response curves for luteal tissue from inadequate CL (Groups 1, 2 and 5) on both day 4 (Fig. 7A) and day 12 (Fig. 7B) of the luteal phase were not significantly different (EC_{50} , 1.0 IU hCG/ml; Table 4). In contrast, EC_{50} concentrations for adequate CL (Groups 3 and 4) were 10-fold lower on both days 4 and 12 (EC_{50} , 0.1 IU hCG/ml; Table 4; $P < 0.05$).

Discussion

Ovulatory response to GnRH and luteal lifespan

Our data have confirmed and extended a number of earlier studies showing that bolus injections of GnRH (Crighton *et al.* 1973, 1975, McNeilly *et al.* 1981) or multiple frequent injections of low doses of GnRH (Crighton *et al.* 1975, McLeod *et al.* 1982a, 1982b) induced ovulation in a proportion of ewes during seasonal anoestrus, but that the resulting CL were smaller, and produced less progesterone than those of the normal luteal phase of the cycle (McNeilly *et al.* 1981, O'Shea *et al.* 1984). All four GnRH-treatment regimens used induced ovulation during anoestrus, although the proportion of ewes ovulating in response to the different treatments did not differ significantly between groups (Table 1). Only ewes in Group 3 (breeding season) and Group 4 (anoestrous animals pretreated with progestagen and given an increasing frequency of GnRH injections) had profiles of progesterone secretion consistent with adequate luteal function (Fig. 2A).

Interestingly, progestagen pretreatment prior to an ovulatory stimulus did not result in the formation of adequate CL in anoestrous Welsh Mountain ewes (Group 2 vs Group 1; Table 1). This contrasts with many other studies in a number of different sheep breeds, using a range of ovulatory stimuli (e.g. GnRH, pregnant mares' serum gonadotrophin, ram effect during anoestrus). The reasons for the failure of progesterone to promote the formation of adequate CL are unclear. Basal LH levels, LH pulse frequency and LH pulse amplitude were similar in the two groups prior to GnRH treatment, as were the magnitude

Figure 5 Progesterone secretion in response to hCG by ovine luteal tissue *in vitro*. (A) Mid-luteal-phase CL were obtained from a local abattoir and transported to the laboratory within 1 h. Triplicate aliquots of minced mid-luteal tissue were incubated in M199 at 37 °C in the presence of increasing concentrations of hCG (0.1 mIU/ml–100 IU/ml) and incubated for 2 h (●), 4 h (○) or 6 h (▲). Points are means $\pm$ range from two experiments in triplicate. (B) Triplicate aliquots of minced luteal tissue were incubated with shaking in M199 at 37 °C in the absence (○) or presence of 0.1 (●) or 10 (▲) IU hCG/ml for the times shown. Tubes were spun at 5000 *g* for 10 min and medium decanted and assayed for progesterone. Points are means $\pm$ range from two experiments in triplicate.

Figure 6 Progesterone secretion by luteal tissue from the different experimental groups on day 4 (A) or day 12 (B) of the luteal phase, incubated with or without 10 IU/ml hCG or 0.5 mM dbcAMP *in vitro*. Luteal tissue was recovered from the different treatment groups on either day 4 (A) or day 12 (B). Tissue was minced finely and triplicate aliquots were incubated for 2 h in M199 at 37 °C in the presence of 100 IU/ml hCG or 0.5 mM dbcAMP. Open columns, inadequate induced luteal tissue (Groups 1, 2 and 5); hatched columns, adequate induced luteal tissue (Group 4); solid columns, breeding-season luteal tissue (Group 3). Column heights represent means and vertical bars are S.E.M. for *n* animals/group, as shown in the legend to Figure 2.

and duration of the LH surge induced (Table 1), ruling out an effect of progesterone on the pituitary gland. It will be of interest to test whether this refractoriness is a consequence of the deep anoestrus experienced by this breed of sheep at higher latitudes, requiring longer duration of exposure to (and/or higher/lower concentrations of) progestagen to prime the ovary to respond.

Luteal function was defined as 'inadequate' when plasma progesterone levels did not exceed 1.5 ng/ml for at least 2 consecutive days. This broad definition includes both short luteal phases and extended luteal phases with reduced progesterone (Xiao *et al.* 2002). Both types of inadequate luteal phase were present in Groups 1, 2 and 5: (a) 'short' luteal phases, characterized by a decline in progesterone to baseline levels within 8 days and (b) extended luteal phases that were maintained until day 12, but with reduced progesterone secretion (see Fig. 2B). Extended luteal phases were associated with higher

Figure 7 Progesterone secretion by luteal tissue minces from the different treatment groups in response to increasing doses of hCG *in vitro*. Luteal tissue recovered from the different treatment groups on either day 4 (A) or day 12 (B) was minced and triplicate aliquots were incubated for 2 h in M199 at 37 °C in the presence of increasing doses (0.1 mIU–100 IU/ml) of hCG. Tubes were spun at 5000 *g* for 10 min and medium decanted and assayed for progesterone. Values were adjusted for DNA content of cell pellets. Group 1 (●); Group 2 (○); Group 3 (▲); Group 4 (△); Group 5 (□). Points are means ± S.E.M. for *n* animals in each group, as shown in the legend to Figure 2.

plasma prolactin levels, suggesting that prolactin may affect luteal phase length. However, short-term pharmacological suppression of prolactin failed to alter luteal lifespan in anoestrous GnRH-treated (Group 5) ewes (McNeilly & Land 1979) or pituitary stalk-disconnected ewes (Niswender *et al.* 1986), and pharmacological elevation of prolactin failed to prevent luteolysis induced by prostaglandin F_{2α} (Sasser *et al.* 1977). Due to small numbers of CL, a direct comparison of the characteristics of 'short' and 'persistent' inadequate luteal phases in the

Table 4 Dose of hCG required to give half-maximal stimulation (EC_{50}) of progesterone secretion *in vitro* by luteal tissue from each group. Data are means $\pm$ S.E.M.

Group	Day	CL number	EC_{50} concentration (IU hCG/ml)
1	4	2	0.91 ± 0.05
2	4	4	0.97 ± 0.09
3	4	6	$0.11 \pm 0.01^*$
4	4	5	$0.09 \pm 0.02^*$
5	4	4	0.95 ± 0.06
1	12	3	1.06 ± 0.12
2	12	5	0.99 ± 0.11
3	12	7	$0.10 \pm 0.02^*$
4	12	5	$0.09 \pm 0.03^*$
5	12	5	1.11 ± 0.13

* Significantly different from Groups 1, 2 and 5 ($P < 0.05$).

same treatment groups was not possible. However, it would be interesting to study whether persistent 'low-progesterone' CL were nevertheless capable of supporting a pregnancy, and whether luteal lifespan was affected by the presence of developing large ovarian follicle(s) in the same ovary.

Morphology of the CL

Morphologically, the total number of cells per CL increased between day 4 and day 12 for all groups (Table 4). Our data for total cell numbers per CL were similar to those reported by O'Shea *et al.* (1984) and Braden *et al.* (1989), but lower than those reported by Farin *et al.* (1990) in superovulated ewes. Total cell number was highest in day 12 CL from ewes during the breeding season, and was significantly greater in ewes with adequate CL (Group 4) than inadequate CL (Groups 1, 2 and 5). Luteal DNA content (an indirect measure of the number of cell nuclei) correlated well with total cell number per CL (Table 4).

The number of large luteal cells ($> 15 \mu\text{m}$ diameter) was similar in all groups on both days (Table 3), in agreement with Braden *et al.* (1989), but in contrast to the data of O'Shea *et al.* (1984). However, mean luteal cell volume increased significantly from day 4 to day 12 (Group 3), and was significantly greater for CL formed in the breeding season compared with groups with inadequate CL (Table 4), in contrast to the study by O'Shea *et al.* (1984). Mean vascular area was similar for all CL on day 4 (Table 4), but increased 3–4-fold by day 12 in all groups, and was maximal in breeding season CL compared with either adequate (Group 4) or inadequate (Groups 1, 2 and 5) CL (Table 4), emphasizing the importance of the development of the luteal vascular system for adequate CL function (Schams & Berisha 2004, Tamanini & De Ambrogi 2004).

The morphometric methods used in this study were subject to a number of caveats. First, the distribution of cellular components within different regions of the ovine CL is not uniform (Gilbert *et al.* 1990). We attempted to overcome this by processing segments of luteal tissue from different regions (cortex and medulla) of the CL. Each segment was then chopped finely into 1 mm^3 pieces that were

individually immersion-fixed and processed, and then 5–10 of these were selected at random for embedding and processing. Moreover, preliminary experiments comparing the effects of counting different numbers of grid squares per field of view on luteal tissue demonstrated that counting four grids gave similar estimates of mean cell counts and variance to counts of ten or more grids (D. Stirling, unpublished observations), assuring us that our methods were statistically reliable. Indeed, a consistent change in a measured parameter in $n > 4$ animals is reported to have a probability of $P < 0.05$ (Cruz-Orive & Weibel 1990).

Secondly, the effects of fixation cause significant cell shrinkage and affect measurement of vascular space (Dharmarajan *et al.* 1983, 1985). Since luteal weights and the consistency of luteal tissue varied with both treatment group and stage of luteal phase, fixation effects may vary between treatment groups and luteal stage. Perfusion fixation of CL was not possible in this study. Furthermore, because the experiments were performed at different times of the year, morphological comparisons could not be made between CL processed for histology at the same time, under identical conditions.

Thirdly, assumptions of a spherical shape for luteal cells *in situ* may not be valid (T.A. Bramley, unpublished observations).

For these reasons, the morphological data obtained must be treated with some caution. Having said this, many of the changes we observed were generally consistent with previous histological findings (O'Shea *et al.* 1984, Rodgers *et al.* 1984, Braden *et al.* 1989, Farin *et al.* 1990). Furthermore, there was also some measure of internal consistency in our data. Taking the average DNA content of a cell as 6 pg, measurements of total cell number appeared to be underestimated by approximately 25–30%. Rodgers *et al.* (1984) found a ratio of cell counts/DNA content of 97%. In our study, this ratio, though lower, was consistent between the different treatment groups. Interestingly, some of the morphological characteristics of GnRH-induced inadequate CL (fewer LH receptors, lower luteal progesterone content, fewer total cells per CL and reduced luteal cell volume) resemble those seen in CL induced in hypophysectomized ewes (Farin *et al.* 1990). Moreover, Fitz *et al.* (1982) demonstrated that injection of hCG during the luteal phase caused a shift in the size distribution of luteal cells, increasing the proportion of larger cell types. Thus, some of the morphological changes observed in the present study are also suggestive of an inability of GnRH-induced CL to respond to the luteotrophic effects of LH *in vivo*.

Luteal LH responsiveness *in vivo*

Ewes injected with a bolus of exogenous oLH on day 3 or day 11 of the luteal phase during the breeding season demonstrated a rapid and significant increase in plasma progesterone levels (Fig. 3A and D; note the 10-fold difference in progesterone levels between days 3 and 11).

A similar progesterone response was observed for ewes in Group 4 (adequate CL) on both days (Fig. 3B and E). In contrast, oLH injection of ewes in Groups 1, 2 and 5 (inadequate CL) failed to elicit a significant progesterone response, despite generating an LH pulse of similar magnitude (Fig. 3C and F). This observation was confirmed serendipitously in ewes that had a spontaneous peak of endogenous LH during the 4 h blood-sampling window (Fig. 4). Endogenous LH peaks were similar in magnitude and duration to oLH peaks generated by injection of exogenous LH in groups of ewes with adequate or inadequate CL. However, whereas a rise in plasma progesterone occurred of similar magnitude to that produced in response to exogenous LH injection following an endogenous LH pulse in ewes with an adequate CL (Groups 3 and 4), no significant response was observed to either exogenous or endogenous LH peaks in ewes with an inadequate CL (Groups 1, 2 and 5). Furthermore, the inability to respond to an LH pulse *in vivo* was apparent on day 3 in ewes that were destined to form an inadequate CL (Figs 3B, 3E, 4A and 4B), despite the fact that progesterone secretion *in vivo* was not distinguishable from that of adequate CL at this stage (Fig. 2). This suggests that the steroidogenic potential of these CL was already compromised by day 3. Similar findings have been reported in Romney Marsh ewes (on days 4–6 after ovulation; Hunter *et al.* 1988, Southee *et al.* 1988a), in Corriedale ewes (on days 8, 9 and 10; O'Shea *et al.* 1984, Rodgers *et al.* 1984) and in Scottish Blackface ewes (on day 5; McNeilly *et al.* 1981). We also found that luteal progesterone content was significantly reduced in the three groups of ewes with inadequate CL compared with those with adequate CL (Table 2). However, in contrast to the data of McNeilly *et al.* (1981) and Hunter *et al.* (1988), luteal progesterone content was higher in both groups with adequate CL on day 4 than later in the luteal phase (Table 2). This may reflect upregulation of steroidogenic enzymes (with a consequent increase in steroid synthesis) prior to the establishment of an adequate luteal vascular system to remove synthesized steroids into the general circulation.

Luteal LH responsiveness *in vitro*

Previous studies of progesterone secretion by ovine luteal tissue *in vitro* have used quite different tissue preparations and/or incubation conditions. Thus, Braden *et al.* (1989) and Chemineau *et al.* (1993) studied progesterone secretion by enzymically dispersed luteal cells, whereas Hunter *et al.* (1988) used luteal slices and McNeilly *et al.* (1981) studied luteal minces. Moreover, the incubation media used, the amount of tissue or number of cells per incubation, and the duration of incubation used (from 3 to 12 h) varied markedly between studies. The present results differ from those of previous studies in a number of important respects. First, we used hCG in preference to oLH, as Hunter *et al.* (1986, 1988) have shown that ovine luteal tissue binds hCG with an affinity 3–30-fold higher

than that of oLH. Secondly, we used luteal minces, as the conditions used to disperse luteal tissue (1) significantly reduced luteal LH receptor levels, possibly compromising steroidogenic response *in vitro* (T.A. Bramley and G.S. Menzies, unpublished observations), and (2) disrupted cell–cell communication, which may be important for normal progesterone secretion (Harrison *et al.* 1987, Del Vecchio *et al.* 1994, Grazul-Bilska *et al.* 2001). Under our incubation conditions, progesterone secretion increased linearly with duration of incubation (both in the absence and presence of hCG) for up to 3 h (Fig. 5A). However, basal progesterone secretion increased progressively with increasing duration of incubation, leading to an apparent flattening of the dose–response curve (Fig. 5B). These factors make direct comparison of our data on steroid secretion and hormonal responsiveness with data obtained by other groups difficult, but may account for the failure of some studies (Braden *et al.* 1989, Chemineau *et al.* 1993) to demonstrate a significant progesterone response to LH *in vitro*.

Studies of *in vitro* progesterone secretion by CL minces from tissue recovered following the different treatments on days 4 or 12 revealed some interesting points. First, basal progesterone secretion by CL of all groups was significantly higher on day 4 than on day 12 (Fig. 6), in line with luteal progesterone content (Table 2). In contrast, maximal response to hCG or to dbcAMP was not significantly different at either stage or between different treatment groups (Fig. 6). However, although maximal response to hCG was similar for the groups with adequate and inadequate CL, we observed a dramatic right-shift in the dose–response curves for hCG in inadequate CL (Groups 1, 2 and 5; Fig. 7) compared with breeding season CL (Group 3) and adequate induced CL (Group 4), resulting in a significant 10-fold difference in EC₅₀ for hCG for adequate and inadequate luteal tissue that was apparent in both day 4 and day 12 (Fig. 7, Table 4). This is in line with our *in vivo* data that showed that CL that are inadequate, or are destined to become inadequate, failed to respond to a pulse of LH with increased progesterone secretion (Figs 3 and 4). Although LH receptor numbers were reduced in inadequate GnRH-induced CL relative to adequate CL groups (Table 2; see also McNeilly *et al.* 1981 and Hunter *et al.* 1988), inadequate luteal tissue still responded maximally to both dbcAMP and high doses of hCG (Figs 6 and 7). The change in EC₅₀ suggested a change in LH/hCG-binding affinity; however, there were no significant differences in K_a for ¹²⁵I-hCG binding to luteal homogenates from the different treatment groups on days 4 or 12 (see above). Finally, differing luteal sensitivity could not be accounted for by differing LH receptor occupancy (Table 2).

In conclusion, we have shown that Welsh Mountain ewes can be induced to ovulate during anoestrus using different GnRH-injection protocols. However, the CL so formed (1) are abnormal morphologically and biochemically, and show characteristics suggestive of inadequate

luteinization and/or LH stimulation, (2) exhibit a markedly diminished progesterone response to oLH injection *in vivo* and (3) demonstrate a markedly reduced sensitivity of progesterone secretion *in vitro* to hCG (an order of magnitude), but show no change in maximal response to either dbcAMP and hCG, suggestive of a profound but subtle defect in the efficacy of LH receptor–second messenger coupling.

Thus, in addition to their well-established premature sensitivity to uterine prostaglandin $F_{2\alpha}$, we have shown that ovulation induced by GnRH injection during anoestrus results in the formation of CL that show defective coupling of LH receptors to downstream intracellular signal transduction pathways. It will be of interest to examine the effects of luteotrophic and luteolytic agents that are known to activate different luteal cell signalling pathways (Niswender 2002, Davis and Rueda 2002) on the responsiveness of these induced CL, *in vivo* and *in vitro*, and thereby elucidate the cause of their reduced LH sensitivity.

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