Maternal pituitary gonadotroph function in relation to GnRH receptor and LH β mRNA content during pregnancy in ewes

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To investigate the mechanism controlling the fall in maternal pituitary responsiveness to GnRH, LH synthesis and pituitary GnRH receptor content during pregnancy, maternal pituitaries were collected from sheep on days 35, 45, 60, 90, 110, 125 and 135 of pregnancy. Circulating steroids and gonadotrophins were determined in blood samples collected from these ewes immediately before death. Pituitary blocks from each ewe were perfused with either medium alone (control) or medium supplemented with oestradiol, oestradiol plus progesterone or oestradiol plus RU486, for 150 min before administration of two 15 s GnRH pulses 90 min apart. The amounts of mRNA encoding LH β and GnRH receptor were determined in pituitary tissue fragments snap-frozen in liquid N₂ at the time of collection from the ewes. While basal LH secretion fell during pregnancy, pituitary responsiveness to GnRH remained high (up to seven times basal LH concentrations). After day 90, the first GnRH pulse elicited LH peaks equivalent to the LH peaks produced by the second GnRH pulse. Therefore, GnRH self-priming was not evident possibly because the pituitaries were constantly primed by increased concentrations of maternal oestradiol. Around day 90, circulating concentrations of progesterone rose from 7.8 ± 1.5 to 12.2 ± 3.8 ng ml⁻¹. Up to day 60, oestradiol in the perfusion buffer had stimulatory effects on LH secretion although this was reduced by RU486. By day 125, the content of mRNA encoding LH β had declined during pregnancy to 7% of the content on day 35, although the content of mRNA encoding GnRH receptor remained unchanged. From these data, there appears to be a transitional period at around day 90 of gestation when pituitary sensitivity to steroids *in vitro* is lost together with detectable GnRH self-priming. In conclusion, the marked decline in pituitary amounts of mRNA encoding LH β , but not in GnRH responsiveness or expression of GnRH receptor, after day 45 of pregnancy suggests that the principal effect of pregnancy on gonadotroph function is mediated via a mechanism other than reduced pituitary amounts of GnRH receptors. Two possible mechanisms are (1) a reduction in GnRH output leading to lowered LH synthesis, or (2) the presence of an inhibitory factor with a short half-life in the maternal circulation.

Introduction

Pituitary responsiveness to GnRH is reduced during human gestation with a period of refractoriness to GnRH during the puerperium and lowered circulating gonadotrophin concentrations (Keye and Jaffe, 1976; Reyes *et al.*, 1976; Miyake *et al.*, 1977; Rubinstein *et al.*, 1978; Sowers *et al.*, 1978; Marrs *et al.*, 1981). In pregnant sheep, reduced pituitary responsiveness to GnRH is associated with lowered pituitary LH content and hypoplasia of LH-secreting gonadotrophs (Chamley *et al.*, 1974, 1976; Jenkin *et al.*, 1977; Wise *et al.*, 1986). However, some data suggest that circulating concentrations of LH and FSH in pregnant ewes are not necessarily reduced progressively (Al-Gubory *et al.*, 1994; Jenkin *et al.*, 1977). In the rat

pituitary, the amount of GnRH receptor and pituitary gonadotrophin content during pregnancy are not reduced, although LH release after GnRH infusion is reduced during early, but not late, pregnancy (Reeves *et al.*, 1982; Koiter *et al.*, 1987).

Increasing steroid hormone concentrations during pregnancy in humans and ewes (Thompson and Wagner, 1974; Thomas *et al.*, 1994; Muttukrishna *et al.*, 1995; Challis and Patrick, 1981) may be responsible for the long-term downregulation of GnRH secretion and the subsequent synthesis and release of gonadotrophins. This is supported by evidence that progesterone has oestradiol-dependent inhibitory effects on LH secretion, with the ability to suppress the ovine oestradiol-induced LH surge (Kasa-Vubu *et al.*, 1992). Similarly, in rats, reduced pituitary responsiveness to GnRH during early pregnancy is associated with increased circulating concentrations of progesterone. It has also been suggested that prolactin

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suppresses pituitary responsiveness to GnRH during pregnancy (Smith, 1980). However, we have demonstrated that human prolactin has no suppressive effects *in vitro* on GnRH-induced LH secretion from ovine pituitaries collected during the breeding season (Fowler *et al.*, 1995). Other factors, such as a placental protein that reduces GnRH bioactivity *in vitro* (Siler-Khodr *et al.*, 1989) and gonadotrophin surge-attenuating factor (GnSAF) in serum from pregnant women (Fowler *et al.*, 1995; Fowler and Templeton, 1996), may play a part in the reduced pituitary responsiveness to GnRH during pregnancy.

After parturition in ewes, GnRH pulse frequency and amplitude increase to cyclic amounts (Wise, 1990). While there are no clear data on GnRH pulse frequency in ewes during gestation, after parturition there is a reversal of the decreased amount of mRNA encoding pituitary LH α and β subunit observed during gestation (Wise *et al.*, 1985). Because of the close relationship between GnRH and LH during the ovine oestrous cycle, a decreased GnRH signal during gestation may be responsible for decreased LH production (Levine *et al.*, 1982; Clarke *et al.*, 1987). Evidence for this is provided by studies in hypothalamic-pituitary-disconnected ewes in which the absence of the GnRH signal leads to a reduction in LH synthesis and secretion (Hamernik *et al.*, 1986). In postpartum ewes, there is an increase in the amount of LH secreted in response to exogenous GnRH and an increased pituitary LH content (Jenkin *et al.*, 1977), while the number of pituitary GnRH receptors remains similar (Crowder *et al.*, 1982). Therefore, it is evident that there are considerable difficulties in separating the functional responsiveness to GnRH during pregnancy from other factors, such as decreasing GnRH input, leading to reduced gonadotrophin synthesis.

The aim of the present study was to separate the functional responsiveness of the ovine pituitary to GnRH *in vitro* from the influences of the endocrine milieu and gonadotrophin synthesis *in vivo* at different stages of gestation. To examine fully the responsiveness of pituitaries from pregnant sheep *in vitro*, the effects of two consecutive GnRH challenges, under different steroidal conditions, on subsequent LH secretion were investigated. Treatments tested were: (1) no steroids, (2) oestradiol alone, (3) oestradiol plus progesterone (to determine whether the progesterone is suppressing pituitary responsiveness to GnRH), and (4) oestradiol plus RU486 (mifepristone), a progesterone antagonist. The rationale for the treatment with oestradiol plus RU486 is based on the observation that the progesterone receptor may be involved in pituitary GnRH receptor function independent of progesterone itself (Dudley *et al.*, 1990) and that this can be prevented by treatment with RU486. The results for pituitary function *in vitro* are compared with circulating steroid concentrations and pituitary steady state concentrations of mRNA encoding LH β and GnRH receptor.

Materials and Methods

Pituitaries and serum samples from pregnant ewes

The pituitaries and serum samples were obtained from Scottish Blackface ewes. These animals were being used in a joint Ministry of Agriculture, Fisheries and Food-Scottish

Office Agriculture, Fisheries and Environment Department-funded project, conducted at the Scottish Agricultural College and Rowett Research Institute in Aberdeen, to determine the development of conceptuses derived from normal and perturbed embryos throughout gestation. The perturbed embryos were exposed on day 3 to a uterine environment at day 6 for 3 days before being transferred to an appropriate uterine environment and were carefully matched with controls transferred at the same gestational stages. There were no effects of this treatment on the progression of gestation, fetal growth or fetal development (Sinclair *et al.*, 1996). All ewes were tested for oestrus using teaser rams and received embryos by transfer 6–9 days after oestrus. Once pregnancy was confirmed by ultrasound, the ewes were slaughtered at 35, 40, 60, 90, 110, 125 and 135 days of gestation at the Rowett Research Institute. The serum samples were obtained, by venepuncture, immediately before pentobarbitone overdose. The pituitaries were then excised immediately upon death. Pituitaries were also collected from eight ewes at a local abattoir during the breeding season (four in December and four in March) to act as nonpregnant, seasonal controls.

Anterior pituitary perfusion

The methodology was a modification of that reported by Fowler *et al.* (1994). Immediately upon excision, the posterior pituitaries were discarded and a quarter of the volume of each anterior pituitary was snap-frozen in liquid N₂, while the remaining three quarters of each anterior pituitary was placed into, and transported in, ice-cold, oxygenated Dulbecco's modified Eagle's medium (DMEM; Sigma Chemical Co. Ltd, Poole) containing 25 mmol Hepes l⁻¹, 4.5 g glucose l⁻¹, 3.71 g NaHCO₃ l⁻¹, 10 ml charcoal-treated sheep serum l⁻¹, 10 ml penicillin-streptomycin solution (containing 5 000 U penicillin ml⁻¹ and 5 mg streptomycin ml⁻¹) and 2.5 g BSA l⁻¹ (all Sigma Chemical Co. Ltd). On each occasion, the pituitary tissues from between two and four ewes were individually sliced into 20 strips of approximately 0.5 mm × 1 mm. The 20 strips from each ewe were mixed individually to ensure that chambers did not receive strips from localized areas of the pituitaries. For each ewe, four randomly selected pituitary tissue strips were placed in each of four perfusion chambers.

Thus, each ewe contributed pituitary explants to one control plus three experimental chambers so that up to 16 chambers were occupied. The perfusion buffer was DMEM as defined above. All buffers were oxygenated for 30 min before the start of the experiment, the pH was adjusted to 7.3 and oxygenation was continued throughout the perfusions. The entire perfusion system, including pumps (Minipuls 3[®], Gilson Medical Electronics (France) SA, Villiers-le-Bel), perfusion chambers and bottles of buffer were all maintained at 37°C in a single perspex air incubator (Incubator SI60, Stuart Scientific, Redhill). After perfusion for 120 min at 0.3 ml min⁻¹, during which period all the perfusate was discarded, 20 fractions were collected at 10 min intervals from each chamber into a 16-channel fraction collector (FC204[®], Gilson Medical Electronics (France) SA).

Subsequent to the 120 min stabilization period, all the perfusion experiments followed the same GnRH treatment regimen (Fig. 1). The first three fractions taken before the first

Fig. 1. Experimental design of ovine pituitary perfusions. After perfusion for 120 min, 20 fractions were collected individually at 10 min intervals from each chamber. The first three fractions comprised basal LH secretion. Two 15 s GnRH pulses were administered at 151 and 241 min of perfusion. The treatments were (a) control (Dulbecco's modified Eagle's medium (DMEM) buffer only), (b) 10^{-12} mol oestradiol l^{-1} throughout supplemented with 10^{-9} mol progesterone l^{-1} from 120 min onwards, (c) 10^{-12} mol oestradiol l^{-1} throughout and (d) 10^{-12} mol oestradiol l^{-1} supplemented with 10^{-9} mol RU486 l^{-1} throughout.

GnRH challenge reflected basal secretion. Two pulses of 10 ng GnRH (Fertagyl: Intervet UK Ltd, Cambridge) ml^{-1} DMEM buffer were then administered for 15 s, 1 min into the fourth and thirteenth 10 min fractions (at 151 min and 241 min of each perfusion). The GnRH-DMEM solutions were made up 5 min before the first pulse, divided into two aliquots and stored in a refrigerator. A 5 ml aliquot of GnRH-DMEM solution was placed in the incubator 2 min before each pulse to equilibrate to 37°C. An excess of concentrated stock aliquots (10^{-4} mol l^{-1}) of oestradiol, progesterone and RU486 were made up before the start of the study and stored at -40°C. The same batch of DMEM powder was used throughout the study.

Repeatability of perfusion technique

To assess the repeatability of the perfusion data, pituitary fragments from four ewes, ranging between 45 days and 135

days of gestation, were perfused in duplicate, occupying eight chambers under the four experimental conditions on each occasion.

Determination of amounts of pituitary mRNA encoding GnRH receptor and LH β

The snap-frozen pituitary quarters were stored at -70°C until the end of the study and then dispatched to the MRC Reproductive Biology Unit, packed in dry ice, for the determination of amounts of mRNA encoding LH β and GnRH receptor, as described by Brooks *et al.* (1992, 1993). Briefly, total RNA was extracted from the remaining pituitary tissue and 15 μ g samples were hybridized with ^{32}P -labelled cDNA probes for GnRH receptor (Brooks *et al.*, 1993) and LH β (Brooks *et al.*, 1992). The efficiency of loading was measured by reprobing the membranes with a rat 18S ribosomal RNA oligonucleotide probe. The intensity of the bands was

quantified using a phosphorimaging system (Phosphorimager 425, Molecular Dynamics, Sevenoaks) and changes in specific mRNA content were expressed relative to 18S rRNA concentrations (arbitrary units). In each case, all measurements were made at random, at the same time with the same labelled probe and carried out in duplicate. The whole process was repeated and the means of each duplicate measurement were compared. No qualitative differences were observed.

Hormone assays

Concentrations of LH in DMEM from ovine perfusions were determined using a homologous ovine radioimmunoassay, as described by Fowler *et al.* (1990). The sensitivity and intra-assay and interassay coefficients of variance were 1.1 ng LH ml⁻¹ (NIDDK-oLH-S23; National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), Bethesda, MD), 6.5% and 15.2%, respectively. Concentrations of LH, FSH and prolactin in the serum collected from the sheep were determined using homologous ovine radioimmunoassays, as described by McNeilly and Andrews (1974), McNeilly *et al.* (1976) and Baird *et al.* (1981). The sensitivity and intra-assay and interassay coefficients of variance were 0.3 ng LH ml⁻¹ (NIDDK-oLH-S23), 9.3% and 17.3%, respectively; 4.9 ng FSH ml⁻¹ (NIDDK-oFSH-S14), 6.1% and 11.4%, respectively; and 0.4 ng prolactin ml⁻¹ (NIDDK-oPRL-S13), 5.1% and 8.9%, respectively. Commercial kits were used to determine oestradiol and progesterone concentrations in the serum samples, as described by Fowler *et al.* (1990).

Statistical analysis

The results for each ewe, under the four experimental conditions, were only included if the perfusion data set was complete (that is, if all data for each ewe under all four experimental conditions were available). The data were rejected where LH secretion decreased below 80% of basal LH secretion at any time. In two instances, GnRH administration had no effect under any of the four experimental conditions; these data were also excluded. In addition, all data from two of the ewes were excluded when flow through one chamber failed on each occasion. Only in cases where these conditions of complete data sets were fulfilled were data for circulating endocrine and pituitary mRNA encoding LH β and GnRH receptor included in the analysis. This rigorous selection procedure ensured that all data sets were matched and contained data from the same ewes under all four experimental conditions.

Once the data selection process had been completed, there remained complete, balanced, perfusion, serum and pituitary content data sets for five ewes from the breeding season and for seven, five, four, five, three, five and five ewes from days 35, 45, 60, 90, 110, 120 and 135 of gestation, respectively. Perfused secretion of pituitary LH was calculated separately for each perfusion chamber, expressing secretion as a percentage of initial secretion over the first 30 min from the same chambers (that is, as a percentage of basal LH secretion). This enabled responses to GnRH to be calculated relative to unstimulated output, controlling for variation in the amount of tissue present in each chamber. Using tissue from the same

animal for control and experimental chambers accounted for individual variability. Initially, analysis was based on grouping the secretion data from pituitary explants from all sheep under each experimental condition by elapsed time separately for each stage of gestation. These data were analysed by two-way ANOVA (time and treatment) with repeated measures. Subsequently, for each individual ewe, the peaks in LH secretion under the three experimental conditions were calculated as percentages of the relevant control LH peaks and compared using two-way ANOVA (treatment and stage of gestation), while within-treatment differences in response to successive GnRH pulses were assessed using the Bonferroni–Dunn post-hoc test. Overall differences in serum hormone concentrations and content of pituitary mRNA were assessed using one-way ANOVA for stage of gestation. Serum hormone concentrations, content of pituitary mRNA and basal and peak LH concentrations were also compared using correlation, calculating significance values using the Fisher's *r* to *z* to determine the significance of the correlation coefficients.

The repeatability of the perfusion technique was assessed by paired *t* test of data where tissues from four ewes (320 pairs of LH concentrations) were perfused in duplicate chambers. The significance of the correlation between the duplicate chambers was assessed using a *z* test. Values are presented as means $\pm$ SEM.

Results

Repeatability of perfusion technique

To compare the repeatability of the technique, pituitary fragments from four ewes ranging between 45 days and 135 days of gestation were perfused in eight chambers under the four experimental conditions. When data for these double sets of perfusions were compared by *t* test, the mean difference between the duplicate chambers was -2.1% ($\pm 95\%$ confidence intervals were -10.2 to $+5.9\%$) and was not significant ($P = 0.610$). The data were highly correlated with a *z* statistic of $P < 0.0001$.

Pituitary function in vitro

As can be seen from the sample perfusion data shown (Figs 2–4), changes occurred in the behaviour of the pituitary fragments *in vitro* before and during gestation. During the breeding season and during the first half of gestation, the response to the second GnRH pulse produced greater LH peaks than the first GnRH pulse, indicating the occurrence of GnRH self-priming. Typically, gonadotroph responsiveness to GnRH was increased by the addition of oestradiol in particular (Figs 2b and 3b). However, the co-administration of RU486 with oestradiol reduced the stimulatory effect of oestradiol alone (Figs 2c and 3c). The increase in LH after the first GnRH pulse was almost constant in ewes administered oestradiol alone, and was boosted by the second pulse (Fig. 3b). However, during the latter part of gestation, the pituitary tissue appeared to be in a continuously primed state, exhibiting marked responses to both GnRH pulses (Fig. 4). The addition of steroids had little effect on the responses of these gonadotrophs to GnRH

Fig. 2. LH secretion by perfused pituitaries collected from five ewes slaughtered randomly during the breeding season: effect of (a) continuous oestradiol (10^{-12} mol l^{-1}) plus acute progesterone administered at the start of fraction collection (∇ ; 10^{-9} mol l^{-1}); (b) continuous oestradiol (10^{-12} mol l^{-1}) alone; and (c) continuous oestradiol (10^{-12} mol l^{-1}) plus continuous RU486 (10^{-9} mol l^{-1}) on responses to two 15 s pulses of 10 ng GnRH ml^{-1} ($\blacktriangledown$). The SEM range for control secretion (Dulbecco's modified Eagle's medium (DMEM) only) is shown by the shaded areas. Results are expressed as percentages of basal LH secretion for each treatment. Significance values (ANOVA, $P < 0.05$) refer to maximal LH secretion following each GnRH pulse: ^asignificantly different from basal LH secretion; ^bsecond LH peak is significantly higher than first LH peak (GnRH self-priming); and ^cexperimental LH peaks are significantly different from control (DMEM only) LH peaks.

Fig. 3. LH secretion by perfused pituitaries collected from seven ewes on day 35 of pregnancy: effect of (a) continuous oestradiol (10^{-12} mol l^{-1}) plus acute progesterone administered at the start of fraction collection (∇ ; 10^{-9} mol l^{-1}); (b) continuous oestradiol (10^{-12} mol l^{-1}) alone and (c) continuous oestradiol (10^{-12} mol l^{-1}) plus continuous RU486 (10^{-9} mol l^{-1}) on responses to two 15 s pulses of 10 ng GnRH ml^{-1} ($\blacktriangledown$). The SEM range for control secretion (Dulbecco's modified Eagle's medium (DMEM) only) is shown by the shaded areas. Results are expressed as percentages of basal LH secretion for each treatment. Significance values (ANOVA, $P < 0.05$) refer to maximal LH secretion after each GnRH pulse: ^asignificantly different from basal LH secretion; ^bsecond LH peak is significantly higher than first LH peak (GnRH self-priming); and ^cexperimental LH peaks are significantly different from control (DMEM only) LH peaks.

Fig. 4. LH secretion by perfused pituitaries collected from five ewes on day 135 of pregnancy: effect of (a) continuous oestradiol (10^{-12} mol l^{-1}) plus acute progesterone administered at the start of fraction collection (∇ ; 10^{-9} mol l^{-1}), (b) continuous oestradiol (10^{-12} mol l^{-1}) alone and (c) continuous oestradiol (10^{-12} mol l^{-1}) plus continuous RU486 (10^{-9} mol l^{-1}) on responses to two 15 s pulses of 10 ng GnRH ml^{-1} ($\blacktriangledown$). The SEM range for control secretion (Dulbecco's modified Eagle's medium (DMEM) only) is shown by the shaded areas. Results are expressed as percentages of basal LH secretion for each treatment. Significance values (ANOVA, $P < 0.05$) refer to maximal LH secretion after each GnRH pulse: ^asignificantly different from basal LH secretion; ^bsecond LH peak is significantly higher than first LH peak (GnRH self-priming); and ^cexperimental LH peaks are significantly different from control (DMEM only) LH peaks.

(Fig. 4a, b) and the co-administration of RU486 with oestradiol (Fig. 4c) caused only a slight reduction in pituitary sensitivity to GnRH.

To compare the effects of stage of gestation on gonadotroph sensitivity to GnRH under all four experimental conditions, the LH peaks in response to each GnRH pulse were calculated separately for each treatment (Fig. 5; this figure shows the response to GnRH in terms of peak LH expressed relative to basal LH secretion, not the absolute LH values). Under control conditions (Fig. 5a), the gonadotrophs produced greater responses to GnRH, relative to basal LH secretion, as gestation continued. Because of this, the phenomenon of GnRH self-priming was apparently lost after day 90, with both the first and second LH peaks reaching values observed only after the second GnRH pulse during the breeding season and early gestation. This pattern remained the same when the gonadotrophs were exposed to combinations of steroids and RU486 (Fig. 5b–d). When these data were expressed relative to control LH peaks, a clearer pattern emerged.

Exposure of the gonadotrophs to oestradiol, and to a lesser extent oestradiol plus progesterone, resulted in an increase in the size of the LH peaks produced by the first (unprimed) and second (primed) GnRH pulses up to and including day 60 of gestation (Fig. 6a, b). From day 90 onwards, treatment with oestradiol or oestradiol plus progesterone had no significant effect on the magnitude of the LH peaks. The marked stimulatory effect of oestradiol on gonadotroph responsiveness to both GnRH pulses of pituitary slices from nonpregnant ewes and those in the first half of gestation (days 35–60) was significantly reversed by the co-administration of RU486 (Fig. 6c). During the second half of gestation, RU486 treatment had no significant effect on the magnitude of the LH peaks.

To look more closely at the differences between the first and second LH peaks as an indication of GnRH self-priming, the value in response to the second pulse was divided by the value in response to the first pulse for each ewe, under each experimental condition, and treatment means were calculated. These data were grouped according to whether, in the control groups, the second LH peaks were significantly higher than the first LH peaks (for ewes in the breeding season, and at days 35–90 and days 110–135 of gestation; Table 1). None of the treatments at any stage of pregnancy, or during the breeding season, had any significant effect on the ratio between the first and second LH peaks (as an indication of the magnitude of GnRH self-priming).

Pituitary mRNA encoding LH β and GnRH receptor

Basal LH secretion *in vitro* fell sharply (ANOVA, $P < 0.001$) between day 45 and day 90 of gestation (from 3.5 ± 0.4 to 0.6 ± 0.1 ng ml^{-1} ; Fig. 7a), a trend that closely mirrored the amount of mRNA encoding LH β detected in the pituitary tissues (which fell from 4.5 ± 1.2 to 0.6 ± 0.4 units, ANOVA, $P < 0.001$; Fig. 7b). However, expression of mRNA encoding GnRH receptor was variable and similar throughout gestation (ranging from 0.040 ± 0.009 to 0.074 ± 0.014 units), apart from on day 90 and day 110 of gestation, when increased values of mRNA encoding GnRH receptor were recorded (0.126 ± 0.028 and 0.107 ± 0.028 units, respectively; ANOVA, $P < 0.05$; Fig. 7c).

Fig. 5. Responsiveness to GnRH pulses after treatment (a) under control conditions, (b) with continuous oestradiol plus acute progesterone, (c) with continuous oestradiol alone, and (d) with continuous oestradiol plus continuous RU486, of pituitaries collected from ewes slaughtered randomly during the breeding season and on seven occasions between days 35 and 135 of pregnancy. LH secretion is shown in response to the first (□) and second (■) 15 s pulse of 10 ng GnRH ml⁻¹. Results are expressed as percentages of basal LH secretion under the relevant treatment conditions. *Significant differences between the first and second GnRH pulses (ANOVA, $P < 0.05$).

Fig. 6. Effects of treatment with (a) continuous oestradiol plus acute progesterone, (b) continuous oestradiol alone and (c) continuous oestradiol plus continuous RU486 on responsiveness to GnRH pulses of pituitaries collected from ewes slaughtered randomly during the breeding season and on seven occasions between days 35 and 135 of pregnancy. LH secretion is shown in response to the first (□) and second (■) 15 s pulse of 10 ng GnRH ml⁻¹. Results are expressed as percentages of the first and second control LH peaks (Dulbeccos modified Eagles medium (DMEM) only). The horizontal line denotes equality with control LH peaks. Significance values (ANOVA) denote differences between pituitary responses to GnRH before and after day 60.

Circulating hormone concentrations

Circulating concentrations of both oestradiol and progesterone rose steadily during gestation (oestradiol: from 22.1 ± 1.5 to 36.1 ± 2.6 pg ml⁻¹, ANOVA, $P < 0.001$; progesterone: from 5.3 ± 0.5 to 14.6 ± 2.5 ng ml⁻¹, ANOVA, $P < 0.001$), with progesterone, in particular, significantly increased from day 100 onwards (Fig. 8a, b). In contrast, circulating concentrations of LH and FSH fell gradually during pregnancy (LH: from 1.41 to

Table 1. Mean ($\pm$ SEM) ratios between first and second LH peak values resulting from the administration of two 15 s pulses of 10 ng GnRH ml⁻¹ to perfused pituitaries collected from ewes slaughtered randomly during the breeding season and on seven occasions between days 35 and 135 of pregnancy, grouped according to the presence or absence of apparent GnRH self-priming

Stage	Treatment			
	Control	10 ⁻¹² mol oestradiol l ⁻¹ + 10 ⁻⁹ mol progesterone l ⁻¹	10 ⁻¹² mol oestradiol l ⁻¹	10 ⁻¹² mol oestradiol l ⁻¹ + 10 ⁻⁹ mol RU486 l ⁻¹
Breeding season	195.7 $\pm$ 21.5*	177.3 $\pm$ 7.7*	249.9 $\pm$ 40.2*	280.4 $\pm$ 42.2*
Days 35–90	222.8 $\pm$ 20.9*	223.6 $\pm$ 31.3*	230.3 $\pm$ 106.2*	257.4 $\pm$ 50.9*
Days 110–135	119.8 $\pm$ 20.1	131.1 $\pm$ 21.4	123.3 $\pm$ 25.1	157.1 $\pm$ 35.3

No treatment ratios were significantly different from control (ANOVA).

*Difference between the first and second LH peak value was significant ($P < 0.05$, ANOVA).

0.72 ng ml⁻¹, ANOVA, $P > 0.05$; FSH: from 1.2 to 0.49 ng ml⁻¹, ANOVA, $P < 0.001$; Fig. 8c, d). Despite a 50% drop in mean concentrations of circulating LH, marked variation in amounts between individuals resulted in a lack of statistical significance overall. Changes in circulating concentrations of prolactin were not statistically significant (Fig. 8e), but involved a slight decrease up to day 110 (decreasing from 101.5 $\pm$ 2.8 to 41.2 $\pm$ 20.2 ng ml⁻¹) and a sudden increase during late pregnancy (to 180.0 $\pm$ 32.0 on day 125 and 138.0 $\pm$ 57.8 ng ml⁻¹ on day 135 of gestation; ANOVA, $P > 0.05$). Again, considerable individual variation in circulating concentrations was observed.

Discussion

We have clearly shown that the responsiveness *in vitro* of ovine pituitaries to GnRH (in terms of the increase in LH released by GnRH pulses) does not decrease during pregnancy. Indeed, the increase in LH over basal LH elicited by the GnRH pulses was highest in the later stages of gestation. However, as gestation proceeded, basal LH secretion *in vitro* decreased fivefold, in line with pituitary LH β message. Thus, the increased responsiveness of the pituitaries to the first GnRH pulse, in particular during the last third of gestation, were more than counter-balanced by decreased LH production. Therefore, despite an apparently greater sensitivity to GnRH *in vitro*, the net output of LH by the pituitaries decreases as pregnancy continues. Changes in LH output *in vivo* would be quantitatively small and, therefore, difficult to detect, whereas comparative changes were detectable in our perfusion model *in vitro*. These findings match the results of studies *in vivo* demonstrating that by day 120 of gestation the pituitaries of pregnant ewes appear to be virtually unresponsive to GnRH (Chamley *et al.*, 1974). Our data consequently support the suggestion made by Chamley *et al.* (1976) that the apparent progressive reduction in pituitary responsiveness to GnRH during gestation could be due to decreased LH synthesis rather than insensitivity to GnRH.

The high correlation coefficient for LH secretion from the four paired tissue experiments carried out to test the repeatability of the technique, together with the lack of any significant difference detected by paired *t* test, suggests that differences in LH responses to various treatments of pituitary tissue blocks from the same animals are physiological rather than

experimental in nature. In addition, the very close agreement between basal LH secretion *in vitro* and pituitary LH β message also indicates the robustness of this perfusion technique as a model for the functional capacity of the pituitary gonadotrophs.

However, although there was no apparent progressive decrease in the responsiveness *in vitro* of the ovine pituitaries to GnRH during gestation, the characteristics of that response changed markedly. The apparent loss of GnRH self-priming after day 90 of gestation probably reflects, in the first instance, the reduction in net gonadotroph LH production and secretion. On close inspection, the first LH peak after day 90, rather than the second, increased by up to three times relative to basal LH output. This occurred at a time when basal LH secretion and amounts of mRNA encoding pituitary LH β were up to five times lower than during the oestrous cycle or early pregnancy. Therefore, the net LH output was actually lower during the latter part of gestation. In a study using pituitary cell monolayer cultures from ewes at different stages of gestation, a similar pattern was observed: the amount of LH released was reduced during late gestation but the percentage of total LH secreted in response to GnRH was higher, especially in the presence of oestradiol (Wise *et al.*, 1986). From these data, it can be concluded that GnRH self-priming is not evident after day 90 because the gonadotrophs are able to secrete a much larger proportion of their LH in response to GnRH. This may reflect some form of steroid priming of the pituitaries, since the circulating concentrations of steroids are high at this time. An alternative possibility may be that the gonadotrophs are simply not producing sufficient LH for GnRH self-priming to be apparent after the second GnRH pulse.

Because the perfusion technique allows only 150 min for release from ovarian and other feedback mechanisms *in vivo* before the first GnRH pulse, these results suggest that there is at least short-term clamping of pituitary function after day 90 of gestation. This clamping could be due to a number of ovarian or placental secretagogues. One candidate is GnSAF bioactivity, which is found in serum from women in the third trimester of gestation (Fowler *et al.*, 1995). Another candidate for causing the downregulation of pituitary function during gestation is LH-release inhibiting factor (LH-RIF), which appears to suppress LH secretion during ovine pregnancy (Al-Gubory *et al.*, 1992). However, conclusive evidence in either case is not currently available.

Fig. 7. (a) Basal LH secretion *in vitro*; and concentrations of mRNA encoding (b) LHB and (c) GnRH receptor in maternal pituitary tissue from ewes slaughtered randomly during the breeding season and on seven occasions between days 35 and 135 of pregnancy. *Significant (ANOVA, $P < 0.05$) reduction in LH secretion *in vitro* or pituitary concentration of mRNA encoding LHB, or increase in pituitary concentration of mRNA encoding GnRH receptor.

Along with our finding that prolactin had no suppressive effects on perfused ovine pituitary responsiveness to GnRH (Fowler *et al.*, 1995), the fact that the circulating concentrations of maternal prolactin were increased only from day 125 onwards indicates that prolactin does not cause reduced pituitary responsiveness to GnRH in pregnant ewes.

Because of the ability of oestradiol and progesterone to inhibit GnRH-induced LH secretion in ewes (Nett *et al.*, 1990; Girmus and Wise, 1992), the rising concentration of circulating steroids during pregnancy may play a role in the reduction of pituitary function during gestation. During the late follicular phase, an abrupt increase in oestradiol initiates the GnRH surge, coincident with the ovine LH surge (Moenter *et al.*, 1990). However, in the present study, it was found that the administration of oestradiol alone, within physiological limits, also had a stimulatory effect on pituitary responsiveness to GnRH during oestrus and pregnancy up to day 60. This effect was reduced, but not abolished, by the co-administration of either physiological concentrations of progesterone or the antiprogestrone RU486 (Fig. 6). These results suggest that it is unlikely to be increased concentrations of oestradiol that reduce pituitary responsiveness to GnRH during gestation. However, circulating concentrations of maternal progesterone are maximal between day 110 and day 135 of gestation. Therefore, in the presence of greater concentrations of oestradiol, the increase in circulating progesterone may be a contributory factor in reduced pituitary function after day 90.

A surprising finding in relation to steroid and RU486 treatment *in vitro* was the lack of significant changes in apparent GnRH self-priming (indicated by the degree of difference in peak LH secreted in response to the first and second GnRH pulses) as a result of adding oestradiol plus progesterone, oestradiol alone or oestradiol plus RU486. The same stock preparation of RU486 was a potent inhibitor of GnRH self-priming in the rats in the study by Byrne *et al.* (1996). This suggests that in the ovine perfusion system, RU486 was acting in a progestagenic, rather than an antiprogestagenic, manner. This effect has been seen in other species, such as the cynomolgus monkey, in which RU486 does not reduce pituitary responsiveness to GnRH (Heikinheimo *et al.*, 1995). In addition, these data underline the observation that an important effect of treatment with oestradiol alone (up to day 60 of gestation) was to stimulate pituitary responsiveness to the first, as well as the second, GnRH pulse. This effect was largely absent when the pituitary blocks were exposed to either oestradiol plus progesterone or oestradiol plus RU486. However, none of these treatments had any significant effect on the abolition of GnRH self-priming that was observed after day 90, suggesting pituitary insensitivity to short-term changes in exposure to steroid hormones.

The fact that GnRH receptor expression did not decrease with falling LH output is an observation that supports our suggestion that decreasing hypothalamic GnRH output is the most probable cause of reduced LH secretion and reduced responsiveness to GnRH *in vivo* during gestation. In ewes treated with GnRH agonist, GnRH receptor binding and expression and LHB expression are reduced compared with controls. Subsequent treatment with oestradiol significantly reverses this effect on GnRH receptor

expression and binding but has no effect on LH β expression (Brooks and McNeilly, 1994). Unfortunately, there do not seem to be any published data on GnRH pulsatility during gestation in ewes. However, in pregnant women, circulating concentrations of GnRH decrease during gestation (Siler-Khodr *et al.*, 1984) and, while GnRH pulse frequency remains unchanged, GnRH pulse amplitude during late gestation is half that seen in early gestation (Petraglia *et al.*, 1994). Taken together, these findings suggest that increased circulating concentrations of oestradiol in pregnant ewes act to maintain GnRH receptor expression and function, but that hypothalamic GnRH output is reduced, leading to low pituitary LH concentrations. Therefore, subsequent administration of GnRH to these animals *in vivo* would be able to evoke only very small increases in LH secretion.

The period around day 90 of gestation appears to be a transition phase in ovine pregnancy. At this time, apparent GnRH self-priming, with the characteristic greater responsiveness to a second than to a first GnRH pulse, and positive feedback of oestradiol *in vitro* are no longer evident. Simultaneously, the circulating concentration of FSH decreases by approximately 50% and pituitary LH synthesis reaches a nadir. In the present study, no sudden changes in oestradiol or prolactin concentrations were observed at this time, although maternal progesterone reached maximal circulating concentrations soon afterwards.

In conclusion, the present study has suggested two main components to the regulation of gonadotrophin secretion and pituitary responsiveness to GnRH during pregnancy in ewes. It is most likely that pituitary function, in terms of LH secretion and LH β expression, is decreased, probably as a result of a reduction in the hypothalamic GnRH signal to the pituitary. Therefore, reduced pituitary responsiveness to GnRH *in vivo* may be a result of a decrease in gonadotrophin synthesis, possibly with contributions from the downregulation of GnRH receptor function and inhibitory ovarian-placental signalling to the pituitary. As a result, the hypothalamo-pituitary-ovarian axis is maintained at a low activation state. In addition, an extrapituitary product, such as LH-RIF or GnSAF, may down-regulate pituitary responsiveness to GnRH *in vivo*, but this would not be evident in our model *in vitro* once the pituitaries have been removed from their endocrine milieu. This possibility could be investigated by culturing or perfusing pituitaries collected from ewes during the oestrous and anoestrous seasons in the presence of serum from ewes at different stages of gestation.

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Fig. 8. Concentrations of (a) oestradiol, (b) progesterone, (c) LH, (d) FSH, and (e) prolactin in maternal serum from ewes slaughtered on seven occasions between days 35 and 135 of pregnancy. Oestradiol, progesterone and FSH showed significant (ANOVA, $P < 0.001$) overall changes in circulating concentrations during the course of pregnancy.

perfusion experiments, and the National Institute of Diabetes and Digestive and Kidney Diseases (Bethesda, MD) and Scottish Antibody Production Unit (Carluke Hospital, Lanarkshire) for the radioimmunoassay material. Pituitaries from ewes during the breeding season were obtained from McIntosh and Donald Abattoir at Portlethen. The authors are grateful to A. J. McMenemy and the workshop of the Department of Bio-Medical Physics for the manufacture of the perfusion chambers used in this study.

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