Ovarian function in ewes after treatment with mifepristone early during the oestrous cycle

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The aim of this study was to determine whether endogenous progesterone regulates synthesis and secretion of luteal oxytocin. In Expt 1, mature ewes ($n = 5$ per group) were assigned randomly to control or mifepristone (RU486) treatment groups. Ewes were injected s.c. twice a day with vehicle or 10 mg RU486 on days 5–7 of the oestrous cycle (oestrus = day 0). On day 8, after an i.v. injection with prostaglandin $F_{2\alpha}$ (250 μ g cloprostenol), venous blood samples were collected at frequent intervals to determine plasma oxytocin concentrations. Plasma oxytocin concentrations of RU486-treated ewes were not significantly different from those of control ewes. In Expt 2, ewes were injected s.c. each day with vehicle or 175 mg RU486 on days 2–5 of the oestrous cycle followed by administration

of prostaglandin $F_{2\alpha}$ on day 6. Four of five RU486-treated ewes showed 'split-oestrus' (oestrous behaviour for 36 h and then again at 84–108 h after the onset of initial oestrus). There was no significant difference in mean plasma oxytocin or progesterone concentrations between treatment groups. The mean masses of mature corpora lutea from control and RU486-treated ewes on day 6 of the oestrous cycle did not differ significantly (394.8 ± 28.8 versus 319.5 ± 48.3 mg). RU486-treated ewes contained mature corpora lutea, new corpora lutea (two of four ewes) and preovulatory follicles (≥ 10 mm, two of four ewes). The average interoestrous interval for RU486-treated ewes was 9 days more than that for control animals (26.2 ± 2.9 versus 17 ± 0.5 days; $P < 0.025$).

Introduction

It is well known that oxytocin is produced by the hypothalamus, stored in the posterior pituitary gland and secreted upon appropriate stimulation. More recently it has been established that oxytocin is also synthesized and secreted by the ruminant corpus luteum during the oestrous cycle (Wathes and Swann, 1982; Flint *et al.*, 1990). Administration of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) to cows and ewes causes a transient increase in plasma oxytocin concentrations, which in some studies has been shown to be associated with induction and promotion of luteal regression (Flint and Sheldrick, 1982; Schallenberger *et al.*, 1984; Flint *et al.*, 1990; Orwig *et al.*, 1994; Salli *et al.*, 2000).

The oxytocin gene is actively transcribed in the bovine and ovine corpus luteum (Ivell and Richter, 1984; Ivell *et al.*, 1985; Jones and Flint, 1988). Although bovine granulosa cells appear to contain a low oxytocin mRNA content, gene transcription is upregulated on the day of ovulation to yield 100-fold more mRNA by day 3 of the oestrous cycle (Ivell *et al.*, 1985). There is no significant positive correlation between the concentrations of oxytocin mRNA and the peptide within the corpus luteum throughout the mid-oestrous cycle

(Fehr *et al.*, 1987). After day 3, luteal concentrations of oxytocin mRNA gradually decrease over the course of the oestrous cycle (Ivell *et al.*, 1985; Jones and Flint, 1988), whereas maximum luteal production of oxytocin in cows and ewes occurs during the mid-oestrous cycle followed by a decrease in production (Sheldrick and Flint, 1983; Abdelgadir *et al.*, 1987). Thus, peak luteal oxytocin production lags behind that of oxytocin mRNA by 3–6 days but does coincide with maximum synthesis of progesterone (Webb *et al.*, 1981; Fehr *et al.*, 1987).

Progesterone receptor mRNA, and the receptor protein have been identified in ovine and bovine preovulatory follicles and corpora lutea (Smith *et al.*, 1995; Bolden-Tiller *et al.*, 2002). Because luteal concentrations of progesterone receptor mRNA and the receptor coincide with the increase in luteal oxytocin mRNA and oxytocin production (Jones and Flint, 1988; Smith *et al.*, 1995), it is conceivable that progesterone may be acting in an autocrine manner to promote oxytocin synthesis and secretion in the ovine corpus luteum. This premise is supported by Voss and Fortune (1993) who reported that progesterone stimulated oxytocin secretion by bovine granulosa cells during the late stages of a 5 day culture. Similarly, when bovine granulosa cells were cultured with progesterone antagonists mifepristone (RU486) and onapristone, oxytocin secretion was significantly reduced and this effect could be reversed by the presence

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of a progestagen (Lioutas *et al.*, 1997). Therefore, the objective of the present study was to determine whether the antagonistic actions of RU486 in the ewe lead to decreased luteal oxytocin synthesis and secretion during the early stages of the oestrous cycle.

Materials and Methods

Animals

Mature Polypay ewes (3–5 years of age, weighing 65–82 kg) with normal oestrous cycles (17 ± 1 days) were assessed for signs of oestrous behaviour twice a day with a vasectomized ram; the first day of observed oestrus was designated as day 0 of the oestrous cycle. Ewes were assigned randomly to treatment groups before the beginning of the study.

Ewes were anaesthetized with an i.v. injection of 5% (w/v) sodium pentothal (Abbott Laboratories, N. Chicago, IL) followed by maintenance of anaesthesia by use of closed circuit inhalation of an oxygen–halothane mixture (Halocarbon Laboratories, River Edge, NJ) to collect luteal tissue from ewes on day 6 and day 8 of the oestrous cycle. The reproductive organs were exposed through a mid-ventral abdominal incision. Luteal tissues were enucleated from the ovaries and immediately stored on ice until weighed. Follicles (5–10 mm in diameter) were left intact, measured *in situ* and details recorded. Intramuscular injections of banamine (Schering-Plough Animal Health Corp., Union, NJ; 50 mg ml^{-1}) and penicillin G procaine (Butler Co., Columbus, OH; $300\,000 \text{ units ml}^{-1}$) were administered immediately after surgery. All experimental procedures and protocols were reviewed and performed in accordance with the institutional Animal Care and Use Committee guidelines at Oregon State University.

Experiment 1

Luteal oxytocin synthesis. Ewes ($n = 5$) were injected s.c. twice a day with 10 mg RU486 (Sigma Chemical Co., St Louis, MO) dissolved in 10 ml corn oil on days 5–7 of the oestrous cycle. Control ewes ($n = 5$) were injected with corn oil only. On day 8, all ewes received an i.v. injection of $\text{PGF}_{2\alpha}$ (250 μg cloprostenol, Schering-Plough Animal Health Corp.). Blood samples were collected at –15 and 0 min to establish the basal secretion of oxytocin, and at 2.5, 5.0, 10.0, 15.0, 20.0, 30.0 and 40.0 min after $\text{PGF}_{2\alpha}$ administration. All blood samples were collected into 10 ml heparinized vacutainer tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ) and EDTA (0.5 mol l^{-1} ; 20 μl) and 1,10 phenanthroline (5 mg ml^{-1} in ethanol; 10 μl) were added immediately to block endogenous oxytocinase activity; the tubes were then stored on ice until use. Blood samples were centrifuged at 1650 g for 12 min at 4°C , and plasma was stored at -20°C until assayed for

oxytocin. Plasma from the samples collected at 15 min before the administration of $\text{PGF}_{2\alpha}$ was also assayed for progesterone by use of radioimmunoassay.

Ovarian morphology. Ewes were assigned randomly to two treatment groups: (i) control ($n = 4$) and (ii) RU486 treatment ($n = 4$) to evaluate the effects of RU486 on ovarian function and morphology. Ewes received s.c. injections twice each day of either 10 mg RU486 dissolved in corn oil or 10 ml corn oil only. Ewes were injected on days 5–7 of the oestrous cycle. On day 8, control and treated animals were anaesthetized and luteal tissue was collected as described above.

Experiment 2

Luteal oxytocin synthesis. Ewes ($n = 5$) were injected s.c. once a day with 175 mg RU486 (Sigma) dissolved in 10 ml corn oil on days 2–5 of the oestrous cycle. Control ewes ($n = 5$) were injected with corn oil only. On day 6, ewes received an i.v. injection of $\text{PGF}_{2\alpha}$ (250 μg cloprostenol). Blood samples were collected at frequent intervals as in Expt 1 for up to 60 min after administration. One sample was collected before administration of $\text{PGF}_{2\alpha}$ to determine plasma progesterone concentrations. All blood samples were collected into 10 ml heparinized vacutainer tubes (Becton Dickinson Vacutainer Systems) and for samples collected for oxytocin analysis EDTA and 1,10 phenanthroline were added immediately and the tubes were then placed on ice for transport to the laboratory. Blood samples were centrifuged at 1650 g for 12 min at 4°C , and plasma was stored at -20°C until assayed for oxytocin and progesterone.

Ovarian morphology. Ewes were assigned randomly to two treatment groups: (i) control ($n = 4$) and (ii) RU486 ($n = 4$) to evaluate the effects of RU486 on ovarian function and morphology. Ewes received s.c. injections of either 175 mg RU486 dissolved in corn oil or 10 ml corn oil only. Ewes were injected once each day on days 2–5 of the oestrous cycle. On day 6, control and RU486-treated animals were anaesthetized, and luteal tissue was collected and follicle diameters measured as described above.

Oxytocin radioimmunoassay. Oxytocin was extracted from 1 ml plasma and measured by radioimmunoassay using methods adapted from Schams (1983) and Abdelgadir *et al.* (1987), using an oxytocin antibody (1 : 7000) generously provided by D. Schams (Technical University of Munich). The mean extraction efficiency was 63% as determined by the addition of [^3H]oxytocin (4 000 c.p.m. per 100 μl ; 2200 Ci mmol^{-1} ; New England Nuclear, Boston, MA). Plasma concentrations of oxytocin determined by radioimmunoassay were corrected for losses due to extraction. Plasma samples of 100 μl per tube were used in the radioimmunoassay. The

sensitivity of the assay was 1 pg ml^{-1} . All samples were analysed in three consecutive assays and the intra- and interassay coefficients of variation were 2.78 and 7.02%, respectively.

Progesterone radioimmunoassay. Plasma concentrations of progesterone were assayed by radioimmunoassay as described by Koligian and Stormshak (1976). Plasma progesterone was extracted from $100 \mu\text{l}$ plasma with benzene:hexane (1:2). $[1,2,6,7-^3\text{H}]$ progesterone ($4000 \text{ c.p.m. per } 100 \mu\text{l}$; $44.5 \text{ Ci mmol}^{-1}$; New England Nuclear) was added to a third tube containing an aliquot of plasma to correct for procedural loss due to extraction. The mean extraction efficiency was 88%.

Extracted samples were quantified by radioimmunoassay using the 337 anti-progesterone-11-BSA (1:2400) provided by G. D. Niswender (Colorado State University). All samples were analysed in two consecutive assays and the intra- and interassay coefficients of variation were 1.9 and 8.4%, respectively. The sensitivity of the assay was 10 pg ml^{-1} .

Statistical analysis

Plasma concentrations of oxytocin were analysed by use of repeated measures ANOVA. Data on luteal masses were analysed by analysis of covariance using the number of corpora lutea as the independent variable, and plasma concentrations of progesterone were analysed by one-way ANOVA.

Results

Experiment 1

Luteal oxytocin synthesis. Treatment of ewes with 10 mg RU486 twice a day on days 5–7 of the oestrous cycle did not result in a significant decrease in luteal oxytocin concentrations compared with those of control ewes after the administration of $\text{PGF}_{2\alpha}$ (Fig. 1). Mean plasma concentrations of progesterone before the administration of $\text{PGF}_{2\alpha}$ on day 8 of the oestrous cycle did not differ significantly ($P = 0.14$) between RU486-treated and control ewes (3.06 ± 0.27 versus $2.16 \pm 0.35 \text{ ng ml}^{-1}$, respectively).

Ovarian morphology. Mean masses of mature corpora lutea in control and RU486-treated ewes did not differ significantly (372.6 ± 32.6 versus $397.2 \pm 27.4 \text{ mg}$, respectively). After administration of $\text{PGF}_{2\alpha}$, all control ewes displayed signs of oestrus within 48 h. However, only three of five RU486-treated ewes displayed signs of oestrus within 48 h after $\text{PGF}_{2\alpha}$ administration; the remaining two ewes had not responded by 96 h after injection of $\text{PGF}_{2\alpha}$.

Fig. 1. Mean ($\pm$ SE) systemic plasma concentrations of oxytocin in ewes treated with corn oil ($\circ$, $n = 5$) or 10 mg RU486 ($\blacksquare$, $n = 5$) twice a day on days 5–7 of the oestrous cycle after administration of prostaglandin $\text{F}_{2\alpha}$ on day 8 (0 min) of the oestrous cycle.

Fig. 2. Mean ($\pm$ SE) systemic plasma concentrations of oxytocin in ewes treated with corn oil ($\circ$, $n = 5$) or 175 mg RU486 ($\blacksquare$, $n = 5$) on days 2–5 of the oestrous cycle after administration of prostaglandin $\text{F}_{2\alpha}$ on day 6 (0 min) of the oestrous cycle.

Experiment 2

Luteal oxytocin synthesis. Treatment of ewes with 175 mg RU486 on days 2–5 of the oestrous cycle did not result in a significant change in $\text{PGF}_{2\alpha}$ -induced luteal oxytocin secretion compared with those of control ewes (Fig. 2). Administration of $\text{PGF}_{2\alpha}$ on day 6 did not cause luteal regression or a shortened oestrous cycle in control or RU486-treated ewes. Control ewes displayed a normal interoestrous interval of 17 ± 0.5 days. After the initiation of treatments, four of five RU486-treated ewes showed signs of a subsequent oestrus, observed as early as 48 h after the end of the original oestrus (Fig. 3). Mean plasma concentrations of progesterone in RU486-treated animals did not differ significantly from those of the control animals (0.92 ± 0.04 versus $0.94 \pm 0.07 \text{ ng ml}^{-1}$, respectively).

Table 1. Ovarian characteristics of RU486-treated and control ewes

Group	(n)	Number of day 6 CL	Average ($\pm$ SE) mass of day 6 CL (mg)*	Number of new CL	Average ($\pm$ SE) follicle diameter (mm) [†]	Average ($\pm$ SE) interoestrous interval [‡]
Control	5	6	394.8 $\pm$ 28.8 ^a	0	7 $\pm$ 0.26	17.0 $\pm$ 0.5 ^a
Treated	5	9	319.5 $\pm$ 48.3 ^a	5	9 $\pm$ 0.58	26.2 $\pm$ 2.9 ^b

*Data were analysed by analysis of covariance.

[†]Two RU486-treated ewes had four follicles with a diameter ≥ 10 mm.

[‡]Interoestrous interval was determined from the onset of the original observed oestrus to the onset of the next normal oestrous cycle.

^{a,b}Means with a different superscript are significantly different ($P < 0.025$).

CL: corpus luteum.

Fig. 3. Oestrous behaviour of ewes treated each day with corn oil (■, $n = 5$) or 175 mg RU486 (□, $n = 5$) during the early luteal phase of the oestrous cycle. Treatments were administered at 48 h (arrow) after the onset of the original oestrus and continued throughout the treatment period. Four of five ewes showed signs of oestrus for a second time between 36 and 60 h after the initial injection of RU486.

Ovarian morphology. Mean masses of mature corpora lutea in control and RU486-treated ewes did not differ significantly (394.8 $\pm$ 28.8 versus 319.5 $\pm$ 48.3 mg, respectively). RU486-treated ewes had new corpora lutea (corpora haemorrhagica) or preovulatory follicles (10 mm in diameter), or both, in addition to mature corpora lutea (Table 1). The average interoestrous interval for RU486-treated ewes was 9 days more than, and was significantly different from ($P < 0.025$) that of the control animals (17 $\pm$ 0.5 versus 26.2 $\pm$ 2.9 days; Table 1).

Discussion

Administration of comparatively low and high dosages of RU486 to ewes during the early to mid-luteal phase of the oestrous cycle did not affect oxytocin synthesis or secretion and did not alter systemic concentrations of progesterone. Regardless of whether the ewes received PGF_{2α} on day 6 or day 8 of the oestrous cycle, both control and RU486-treated ewes responded to exogenous PGF_{2α} with a maximum release of oxytocin detected within 2.5 min after injections. The observed

response to PGF_{2α} was similar to those reported by Orwig *et al.* (1994) and Salli *et al.* (2000). The systemic concentrations of luteal oxytocin are substantially lower on day 6, during the early luteal phase, than on day 8 of the oestrous cycle. Systemic oxytocin concentrations after the administration of PGF_{2α} during the early and mid-luteal phase are also consistent with values observed in the utero-ovarian venous blood of ewes within the same time period (Hooper *et al.*, 1986).

Neither control nor RU486-treated ewes injected with PGF_{2α} on day 6 had a shortened oestrous cycle. Failure of the administration of PGF_{2α} to result in a shortened oestrous cycle may have been due to the refractoriness of the corpora lutea to this eicosanoid early in the luteal phase (Acritopoulou and Haresign, 1980). However, control and RU486-treated ewes that received PGF_{2α} on day 8 of the oestrous cycle showed signs of behavioural oestrus within 48 h.

Although unexpected, the results of the present study indicate that treatment with RU 486 during metoestrus results in a 'split oestrus'. This split oestrus was observed in four of five ewes that received two injections of 175 mg RU486 within 84 h after the onset of initial observed oestrus. This result was not observed in ewes that received the lower dose of RU486 on days 5–7 of the oestrous cycle in Expt 1. Similarly, split oestrus was not reported by Morgan *et al.* (1993) who injected ewes with 2.5 mg RU486 kg⁻¹ each day on days 5–8 of the oestrous cycle. These workers also observed the maintenance of original corpus luteum on day 15 in RU486-treated ewes but no additional ovulations or accessory corpora lutea were detected. In a concurrent study in our laboratory, 150 mg RU486 administered to ewes at the onset of oestrus, and 24 and 48 h later, resulted in the same phenomenon of split oestrus in three of five RU486-treated ewes within 96 h after onset of the original oestrus (A. Wurst, unpublished).

These observations of split oestrus may be attributed to the antagonistic actions of RU486 at the hypothalamus. RU486 is a potent anti-progestin in reproductive tissues, as well as in the central nervous system (Philbert, 1984). In studies conducted in female rat hypothalamus, RU486 bound with very high affinity to progesterone

receptors, particularly in the preoptic nuclei (Pleim *et al.*, 1990). Thus, it is conceivable that RU486 may be binding to progesterone receptors in the hypothalamus, blocking the negative feedback inhibition of endogenous progesterone (Skinner *et al.*, 1999) and encouraging the maturation of the next follicular wave with consequent secretion of oestrogen in sufficient quantities to elicit oestrous behaviour.

Ovarian morphology of ewes that received 175 mg RU486 revealed the presence of mature corpora lutea, new sites of ovulation and preovulatory follicles that occurred in various combinations in individual ovaries. It has been observed that ewes treated with RU486 during the early luteal phase resulted in additional LH surges within hours after the initial ovulatory LH surge (Campbell *et al.*, 2000). These researchers concluded that the subsequent LH surges resulted from the positive feedback action of oestradiol derived from developing follicles, which is consistent with the findings of our morphological studies.

The interoestrous interval was extended in ewes that received 175 mg RU486 each day from day 2 to day 5 of the oestrous cycle. The 9 day extension of the oestrous cycle in these ewes compared with that of the control ewes may be attributed to the formation of new corpora lutea at the split oestrus, which were able to remain functional for the duration of a normal oestrous cycle. However, it is also conceivable that the lifespan of the original corpus luteum was extended. According to Morgan *et al.* (1993), treatment of ewes with RU486 from day 5 to day 8 interfered with the normal timing of luteolysis by causing a delay in the onset of a pulsatile pattern of PGF_{2α} secretion by the uterus. Failure of luteolysis to occur was supported by the finding that plasma concentrations of progesterone were significantly greater in RU486-treated ewes than in control ewes on days 16, 18, 20 and 22 after oestrus.

RU486 administration during the early luteal phase of the oestrous cycle appears to be a powerful antagonist at the hypothalamus, and perhaps the uterus. It has been proposed that progesterone acts in an autocrine manner to regulate its synthesis, ultimately affecting its paracrine actions and regulation of other hormones (Rothchild, 1981, 1996). Although the highest dosage of RU486 used in the present study was apparently able to block the negative feedback of progesterone at the hypothalamo-hypophyseal axis, it was not able to induce or sustain a marked reduction in luteal function. Progesterone has been shown to stimulate oxytocin secretion by granulosa cells *in vitro* (Voss and Fortune, 1993), whereas RU486 suppressed the production of this nonapeptide by cultured granulosa cells (Lioutas *et al.*, 1997). Thus, failure of RU486 to effect a detectable change in luteal oxytocin production in the present study may be attributable to the presence of an insufficient quantity of the progesterone antagonist in the corpus luteum. Further studies are required to examine the

autocrine action of progesterone in regulating ovine luteal oxytocin synthesis using a methodological approach to ensure that the corpus luteum is exposed to an increased quantity of RU486 through mid-oestrous cycle.

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