Increase in concentration of uterine oxytocin receptors and decrease in response to 13,14-dihydro-15-keto prostaglandin $F_{2\alpha}$ in ewes after withdrawal of exogenous progesterone

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Summary. Ovariectomized ewes were treated with progesterone and oestradiol to induce oestrus (day of expected oestrus = day 0) and with progesterone on days 1 to 12. The concentrations of endometrial oxytocin receptors and the 13,14-dihydro-15-keto prostaglandin $F_{2\alpha}$ (PGFM) response induced by oxytocin were measured on days 12, 14, 16 and 18 after the cessation of progesterone treatment on day 12, by a receptor binding assay and direct radioimmunoassay, respectively. During the period of treatment, the concentrations of plasma progesterone were high and remained above 2 ng ml^{-1} until day 13 when they dropped rapidly to less than 0.5 ng ml^{-1} by day 14. The concentrations of oxytocin receptors in endometrium of control ewes were high (820.7 ± 91.7 (SEM) fmol mg^{-1} protein). Treatment with progesterone significantly ($P < 0.01$) reduced the concentrations of the receptors on days 12 and 14 (144.1 ± 65.0 and $200.4 \pm 45.4 \text{ fmol mg}^{-1}$ protein, respectively). The receptor concentrations then increased to relatively high values on day 16 ($1021.4 \pm 216.6 \text{ fmol mg}^{-1}$ protein) and remained high until day 18 ($677.7 \pm 103.4 \text{ fmol mg}^{-1}$ protein). In contrast, the oxytocin-induced PGFM response was low in control ewes ($2.7 \pm 1.5 \text{ ng min}^{-1} \text{ ml}^{-1}$ and $74.2 \pm 29.9 \text{ pg ml}^{-1}$, for the area under the curve and peak concentrations, respectively), and treatment with progesterone did not increase the response on day 12 ($2.2 \pm 0.8 \text{ ng min}^{-1} \text{ ml}^{-1}$ and $99.3 \pm 42.9 \text{ pg ml}^{-1}$; but, on day 14 the response ($13.3 \pm 2.9 \text{ ng min}^{-1} \text{ ml}^{-1}$ and $297.1 \pm 55.2 \text{ pg ml}^{-1}$) was significantly ($P < 0.02$) higher than those observed in the control ewes or day 12 ewes. The oxytocin-induced response declined rapidly after day 14, and by day 16 and 18, the PGFM response induced by oxytocin ($7.9 \pm 2.7 \text{ ng min}^{-1} \text{ ml}^{-1}$ and $188.3 \pm 48.0 \text{ pg ml}^{-1}$; $1.6 \pm 0.3 \text{ ng min}^{-1} \text{ ml}^{-1}$ and $58.9 \pm 11.8 \text{ pg ml}^{-1}$, for days 16 and 18, respectively) was not significantly ($P > 0.05$) different from those in the control ewes or day 12 ewes. These results confirm previous reports suggesting that treatment with progesterone can suppress the concentrations of endometrial oxytocin receptors while simultaneously increasing the PGFM response induced by oxytocin. The results also show that the withdrawal of exogenous progesterone alone is followed by a rapid increase in the endometrial concentration of oxytocin receptors and a reduction in the PGFM response induced by oxytocin.

Keywords: progesterone; oxytocin; receptor; uterus; sheep

Introduction

In sheep, prostaglandin $F_{2\alpha}$ ($\text{PGF}_{2\alpha}$) is the luteolytic agent that can cause the demise of the corpus luteum (Goding 1974). The release of $\text{PGF}_{2\alpha}$ is triggered by the binding of luteal oxytocin (Flint &

Sheldrick, 1983) to its specific receptors in the uterus (Roberts *et al.*, 1976). The release of $\text{PGF}_{2\alpha}$ and the concentrations of oxytocin receptors vary during the oestrous cycle. Temporal studies have shown that the series of intermittent surges in $\text{PGF}_{2\alpha}$ release occur at the onset of luteolysis at about days 14–15 (Fairclough *et al.*, 1980), whereas the concentrations of oxytocin receptors increase gradually to peak values at oestrus on days 16–17 (Roberts *et al.*, 1976; Sheldrick & Flint, 1985). The pulsatile secretion of $\text{PGF}_{2\alpha}$ and the increase in the concentrations of oxytocin receptors coincide with the decline in the peripheral concentrations of progesterone (Sheldrick & Flint, 1985).

It has been reported that progesterone is involved in the regulation of $\text{PGF}_{2\alpha}$ release and of the concentrations of endometrial oxytocin receptors. Short-term treatment with progesterone can reduce the oxytocin-induced $\text{PGF}_{2\alpha}$ response (McCracken *et al.*, 1984; Homanics & Silvia, 1988), whereas prolonged treatment with progesterone will eventually lead to a refractoriness of the uterine PG synthetic mechanism to progesterone inhibition (McCracken *et al.*, 1984; Homanics & Silvia, 1988). Progesterone also decreases concentrations of oxytocin receptors in ovariectomized ewes (Vallet *et al.*, 1990; Lau *et al.*, 1992). Leavitt *et al.* (1985) reported that withdrawal of progesterone treatment from ovariectomized ewes given constant infusion of oestradiol was followed by a rapid recovery of the nuclear oestrogen and uterine oxytocin receptors. However, it is not clear in the study of Leavitt *et al.* (1985) whether the increase in the concentrations of uterine oxytocin receptors was due to the influence of continuous infusion of oestradiol or due to the withdrawal of progesterone or due to both of these factors.

This experiment was undertaken to determine whether withdrawal of progesterone treatment alone would result in a recovery of the concentrations of endometrial oxytocin receptors and an increase in the PGFM response to oxytocin.

Materials and Methods

Animals

Thirty 3–4-year-old Merino ewes were ovariectomized under general anaesthesia at least three months before the start of the experiment.

Pretreatment of animals

All ewes were treated for six days with progesterone and oestradiol to induce oestrus (day of expected oestrus = day 0), as described by Lau *et al.* (1992). The treatment consisted of i.m. injections of 12 mg progesterone (Roussel Uclaf, France) once a day for 4 days at 08:00 h followed by five i.m. injections of 5, 10, 20, 10 and 5 μg oestradiol (Sigma, St Louis, MO, USA), at intervals of 8 h starting at 16:00 h the day after the last injection of progesterone was given. Both progesterone and oestradiol were made up in 1 ml vegetable oil.

Treatment of animals

Treated ewes were randomly allocated to five groups ($n = 6$). Groups 2–5 were given progesterone treatment at 08:00 h and 20:00 h on days 1–12 at various doses (Table 1). The control ewes (group 1) were given injections of the vehicle only. Blood samples (6–8 ml) were taken by venepuncture, from all ewes in groups 1 (control) and 5 (representative for the progesterone-treated ewes) on days 4–18 before the 08:00 h injections. Ewes in groups 2–5 were given an i.v. injection of 10 iu oxytocin (Heriot Agvet, Australia) at 08:00 h, on day 12 (group 2), day 14 (group 3), day 16 (group 4) and day 18 (group 5). Blood samples were taken at –10, –5 and 5, 10, 20, 30, 45 and 60 min from the time of the injection of oxytocin, for the determination of concentrations of 13,14-dihydro-15-keto $\text{PGF}_{2\alpha}$ (PGFM) in plasma. The control ewes were studied at the same time as group 2 ewes. The ewes were hysterectomized under general anaesthesia, on day 12 (groups 1 and 2), 14 (group 3), 16 (group 4) or 18 (group 5), by 1 h after the last blood sample was taken. The uteri were excised and placed onto ice for preparation of endometrial oxytocin receptors.

Progesterone assay

Concentrations of plasma progesterone were measured using a commercial Direct Progesterone [^{125}I] Radioimmunoassay Kit purchased from Farnos Diagnostica (Finland). The sensitivity of the assay was 0.16 ng ml^{-1} .

Table 1. Schedule for progesterone administration to ovariectomized ewes

Day ^a	Time (h)	Progesterone ^b (mg)
1	08:00	0.50
	20:00	0.63
2	08:00	0.75
	20:00	1.00
3	08:00	1.25
	20:00	1.50
4	08:00	2.00
	20:00	2.50
5	08:00	3.00
	20:00	4.00
6	08:00	5.00
	20:00	6.00
7, 8	08:00	8.00
	20:00	8.00
9–12	08:00	12.00
	20:00	12.00

^aDay 0 = day of expected oestrus; ^bmade up in 1 ml vegetable oil.

The crossreactivity of the antisera for progesterone, 11 β -hydroxyprogesterone, 5 α -dihydroprogesterone, 5 β -dihydroprogesterone, hydroxyprogesterone derivatives, pregnen derivatives, corticoids, testosterone and oestrogens was 100, 75, 8.8, 7.1, <0.3, <0.3, <0.01–1, <0.01 and <0.01%, respectively. The intra- and interassay coefficients of variation for a plasma pool collected from pregnant ewes on day 10 of pregnancy were 6.5% ($n = 10$) and 12.6% ($n = 10$), respectively. The correlation coefficient between this assay and the extraction assay used in our laboratory (Fairclough *et al.*, 1975) was 0.996.

Assay of endometrial oxytocin receptors

The endometrial microsomal fractions were prepared and the oxytocin receptor concentrations were measured by a receptor binding assay as described by Lau *et al.* (1992). The endometrial tissues, 3–5 g, containing both the caruncular and intercaruncular endometrium, were homogenized in Tris-HCl (50 mmol l⁻¹, pH 7.6) containing 4 mmol EDTA l⁻¹. The addition of 4 mmol EDTA l⁻¹ was to dissociate any endogenously bound oxytocin. The resulting homogenate was centrifuged at 2100 *g* for 30 min at 4°C. The supernatant was filtered through four layers of surgical gauze and centrifuged at 216 000 *g* for 30 min at 4°C. The pellets were rinsed twice with 5 ml 50 mmol Tris-HCl l⁻¹, pH 7.6 and resuspended in 6–10 ml (equivalent to twice volume of the original tissue weight) of the same buffer and stored at -20°C until assayed. All materials were kept on ice during preparation.

Endometrial microsomal fractions (100 μ l) containing approximately 180–250 μ g protein were incubated for 2 h at room temperature with 100 μ l of [³H]oxytocin (approximately 0.32 pmol in 100 μ l, [Tyrosyl-3,5-³H]oxytocin, 39.3 Ci mmol⁻¹, New England Nuclear, USA) and 100 μ l nonradioactive oxytocin (Calbiochem, USA) containing various amounts of oxytocin ranging from 0 to 4 pmol made up in 50 mmol Tris-HCl l⁻¹. The [³H]oxytocin was made up in the same buffer with the addition of 0.3% BSA and 15 mmol MnCl₂ l⁻¹. Nonspecific binding was measured by the presence of saturating concentrations (40 pmol) of nonradioactive oxytocin in the incubation. The bound and free fractions were separated by adding 200 μ l of human IgG (8 mg ml⁻¹, Commonwealth Serum Laboratories, Australia) and 1 ml of 22% (w/v) polyethylene glycol 6000 (BDH, UK) and centrifuged at 2100 *g* for 30 min at 4°C. The pellets were resuspended in 200 μ l 10 mmol HCl l⁻¹, and the tubes rinsed with another 200 μ l HCl and transferred into scintillation vials containing 5 ml of Emulsifier Safe (Packard, USA). Radioactivity measurements were determined using a Beta counter (Rackbeta 1212, LKB, Sweden), and the c.p.m. were converted to d.p.m. by taking into account the counting efficiency of the instrument. The data were then subjected to Scatchard (1949) analyses to determine the affinity (K_d) and the concentration of oxytocin receptors. The intra- and interassay coefficients of variation for the receptor binding assay were 3% ($n = 4$) and 7% ($n = 4$), respectively. The protein concentrations of the samples were measured by a Bio Rad Protein Kit II (Bio Rad, USA) using a BSA (Fraction V) standard (Sigma, St Louis, MO, USA).

Assay of prostaglandin F_{2a} metabolite (PGFM)

Concentrations of PGFM in plasma were measured by a radioimmunoassay as described by Lau *et al.* (1992). The [³H]PGFM (approximately 8000 c.p.m. in 100 μ l, 13,14-dihydro-15-keto-[5,6,8,9,11,12,14(n)-³H]PGF_{2a}) was

purchased from Amersham (Buckinghamshire, UK). The PGFM antiserum was raised in goats and the cross-reactivity for PGFM, 13,14-dihydro-PGF_{2α}, 15-keto-PGF_{2α}, PGF_{2α}, and C₁₆ urinary metabolite of PGF_{2α} were 100, 0.5, 20, <0.1 and <1%, respectively. Since the antiserum could not differentiate between 13,14-dihydro-15-keto-PGF_{2α} and 13,14-dihydro-15-keto-PGF_{1α}, the results are expressed as PGFM equivalents. The sensitivity of the assay was 62.5 pg ml⁻¹, and the intra- and interassay coefficients of variation were 10% and 16%, respectively. The response to oxytocin stimulus was assessed by the area under the basal-level-adjusted PGFM curve (i.e. total PGFM response) calculated by the trapezoidal rule (Abramowitz & Stegun, 1972) and the peak PGFM concentrations. The basal concentrations were defined as the mean concentrations of the two pre-injection bleeds.

Statistical analysis

The differences between one treatment group and another, in the PGFM response induced by oxytocin, in the K_d values and the concentrations of endometrial oxytocin receptors, were assessed by one-way analysis of variance (ANOVA). All values are presented as means $\pm$ SEM.

Results

Concentration of progesterone in plasma

As the blood samples for determination of concentrations of progesterone in plasma were taken before the 08:00 h injections, the plasma progesterone concentrations for blood samples taken on a particular day represent the lowest plasma progesterone concentrations of the previous day (Fig. 1).

Fig. 1. Concentrations of progesterone in plasma of control (△) and treated (○) ewes ($n = 6$ per group) during the period of treatment (days 1–12) and 6 days (days 13–18) after the cessation of treatment. Treatment was given as i.m. injection of progesterone (see Table 1 for doses). Values presented are means $\pm$ SEM.

After treatment with progesterone, the concentrations of progesterone in peripheral plasma rose rapidly on day 5 and remained above 2 ng ml⁻¹ on days 6–13 (one day after progesterone treatment was withdrawn). The plasma progesterone concentrations dropped rapidly to less than 0.5 ng ml⁻¹ on day 14; progesterone could not be detected after day 16.

Endometrial oxytocin receptors

The percentage of specific binding of [³H]oxytocin to the microsomal proteins dropped from the control of 14.0% ($\pm 2.0\%$) to 3.0% ($\pm 1.9\%$) after 12 days of treatment with progesterone (day 12) and to 2.8% ($\pm 0.6\%$) 2 days after that (day 14). However, the specific binding was restored to

15.7% ($\pm 4.6\%$) and 15.7% ($\pm 3.4\%$) on days 16 and 18 respectively. These percentages of specific binding reflected the concentrations of oxytocin receptors in the various treatment groups (Fig. 2). In contrast, the percentage of nonspecific binding was low, and did not vary among all treatment (maximum group average of $5.5 \pm 0.48\%$).

Fig. 2. Concentrations of oxytocin receptors in endometrium on days 12, 14, 16 and 18 in ovariectomized ewes ($n = 6$ per group) after injections of progesterone on days 1–12. Values presented are means $\pm$ SEM.

*Different from the control at $P < 0.05$.

Scatchard analysis of the binding data yielded linear lines with an average apparent K_d of 2.43 ± 0.22 nmol l⁻¹ for all treatment groups. ANOVA indicated that there was no significant ($P > 0.05$) difference in the apparent K_d values among the different treatments.

The mean concentration of oxytocin receptors of endometrium of the control ewes were high (Fig. 2). Treatment with progesterone for 12 days significantly ($P < 0.01$) lowered the concentrations of oxytocin receptors in the endometrium in ewes on day 12 (i.e. on the day of cessation of treatment) and day 14 (i.e. 2 days after treatment ceased). The concentrations of oxytocin receptors then increased to relatively high values on day 16 and day 18 (4 days, and 6 days, after progesterone treatment stopped, respectively). By this time the receptor concentrations observed in these ewes were the same as those found in control ewes, and were significantly ($P < 0.01$) higher than those found on the day and two days after progesterone treatment ended. The concentrations of oxytocin receptors observed on day 16 were not significantly ($P > 0.05$) different from those observed on day 18.

PGFM response

The total PGFM response was low in the control ewes that received only the injection of the vehicle (Fig. 3a). Treatment with progesterone for 12 days did not significantly ($P > 0.05$) increase the total PGFM response on day 12 (the day of withdrawal of progesterone treatment). However, there was a significant ($P < 0.02$) increase in the total PGFM response on day 14 (2 days after progesterone treatment was withdrawn) compared with those observed in the control and on the day progesterone was withdrawn. There was a gradual decline in the total PGFM response after day 14, and by day 16 (4 days after progesterone treatment was withdrawn) the total PGFM response was not significantly different from those observed in the control or on day 12. The same trend was observed in all treatment groups for the peak PGFM concentrations (Fig. 3b). The

average time taken for the concentrations of PGFM to reach peak values in response to an oxytocin stimulus was $28.5 (\pm 3.7)$ min. There was no significant difference in the timing of occurrence of peak PGFM concentrations among the various treatment groups.

Fig. 3. (a) Total 13,14-dihydro-15-keto-prostaglandin $F_{2\alpha}$ (PGFM) responses; (b) peak concentrations of PGFM observed on days 12, 14, 16 and 18 in ovariectomized ewes after an injection of 10 iu oxytocin. Ewes received no treatment (control) or injections of progesterone on days 1–12 (see Table 1 for doses). Values presented are means $\pm$ SEM.

a,b,c Values with different letters differ at $P < 0.05$.

Discussion

The results of this experiment showed that withdrawal of exogenous progesterone was followed by a rapid increase in the concentrations of oxytocin receptors in the endometrium and a gradual decline in the PGFM response to oxytocin.

The concentrations of oxytocin receptors observed in the two groups of ewes at days 12 and 14 after progesterone treatment were significantly lower than those observed in the control ewes, but were not significantly different from each other. The reductions in the concentrations of oxytocin receptors after treatment with progesterone were primarily due to the inhibitory effect of progesterone on oxytocin receptors (Vallet *et al.*, 1990; Lau *et al.*, 1992). Although progesterone treatment was withdrawn on day 12, the peripheral concentrations of progesterone remained high on day 13 and fell to below 0.5 ng ml^{-1} by 08:00 h on day 14 only. The low concentrations of oxytocin receptors observed on day 14 may be due to the residual effect of progesterone.

In this study, the concentrations of oxytocin receptors were low after 12 days of treatment with progesterone, whereas Vallet *et al.* (1990) reported high concentrations of oxytocin receptors after the same period of progesterone treatment. This apparent contradiction was probably due to the different pretreatment and progesterone treatment regimens used in the two studies. In this study, progesterone was given at increasing doses to mimic the changes in concentrations of progesterone in plasma observed during the oestrous cycle in ewes. With this treatment, progesterone concentrations were increased to above 0.5 ng ml^{-1} at about day 5 of treatment, and, by day 12, the uterus has been exposed to high progesterone concentrations for only 8 days. In contrast, Vallet *et al.* (1990) administered the same dose of progesterone (10 mg twice a day) for 12 days. This treatment could have exposed the uterus to high progesterone concentrations in plasma for the whole treatment period (i.e. 12 days). It is possible that exposure of the uterus to high progesterone concentrations over a certain period could result in the uterine oxytocin receptors becoming refractory to the inhibitory effect of progesterone. This proposal is consistent with the results reported by Vallet *et al.* (1990) indicating that the concentrations of oxytocin receptors in the ovariectomized ewes given progesterone for 5 days were lower than those observed in ewes given progesterone for 12 days. Vallet & Lamming (1990) showed that the concentrations of oxytocin receptors in ovariectomized ewes given progesterone for 8 days were significantly lower than those in ewes given progesterone for 10 days. In addition, preliminary results obtained in our laboratory indicate that the concentrations of oxytocin receptors in ovariectomized ewes were significantly reduced after 14 days of progesterone treatment, but the receptor concentrations were increased after 16 days of progesterone treatment (Lau, Kerton, Gow & Fairclough, unpublished).

The rapid increase in the concentrations of oxytocin receptors in the endometrium two days after the peripheral progesterone concentration has fallen below 0.5 ng ml^{-1} (i.e. day 16) indicated that the increase in concentration of oxytocin receptors was probably due to the removal of the inhibitory effect of progesterone. In a previous report, Leavitt *et al.* (1985) showed that withdrawal of progesterone treatment was followed by an increase in the concentrations of nuclear oestrogen receptors and concentrations of oxytocin receptors in ovariectomized ewes given constant infusion of oestradiol.

In cyclic ewes, the concentrations of oxytocin receptors in the uterus were low on days 12–14 of the oestrous cycle and increased to a peak value on the day of oestrus (days 16–17) (Roberts *et al.*, 1976; Sheldrick & Flint, 1985). The increase in the concentration of oxytocin receptors coincided with the fall in the plasma concentration of progesterone (Sheldrick & Flint, 1985). In light of the observed rapid increase in the concentrations of oxytocin receptors after the withdrawal of progesterone (this study), it is possible that the increase in the concentration of oxytocin receptors at luteolysis may be due to the withdrawal of inhibition by progesterone.

In contrast, the oxytocin-induced PGFM response was significantly higher on day 14 than on day 12 or in the controls. The increase in the oxytocin-induced PGFM response observed on day 14, compared with those observed on day 12, was probably due to the prolonged exposure of the uterus to high concentrations of progesterone. This prolonged exposure of the uterus to progesterone may have resulted in the secretory response of the uterus becoming refractory to the inhibitory effect of progesterone. This proposal is consistent with the results reported by Homanics & Silvia (1988) which indicated that in ovariectomized ewes the PGFM response induced by oxytocin was low after 10 days' treatment with progesterone, whereas the response was significantly increased after 15 days of treatment with progesterone. However, the PGFM response declined over days 16–18. The parallel decline in the PGFM response and peripheral progesterone concentrations over this period suggests that the lower PGFM response on day 18 compared with that on day 16 may be due to the depletion of the precursors for $\text{PGF}_{2\alpha}$ synthesis. This suggestion is consistent with the observation in cyclic ewes that the storage of uterine intraepithelial neutral lipids changes in parallel with the peripheral progesterone concentrations (Boshier *et al.*, 1987).

The findings in this study that the PGFM response induced by oxytocin preceded the rise in the concentrations of endometrial oxytocin receptors at the withdrawal of progesterone treatment

indicate that progesterone may act to control PG release and the regulation of concentrations of oxytocin receptors in endometrium through separate mechanisms. Vallet *et al.* (1990) have suggested that progesterone influences the release of $\text{PGF}_{2\alpha}$ by controlling the factors coupling the oxytocin receptor to PG release. Progesterone induces expression of endometrial mRNA encoding for cyclooxygenase synthesis (Eggleston *et al.*, 1990), and increases the oxytocin-stimulated phosphatidylinositol turnover in the endometrium of sheep (Vallet & Bazer, 1989). However, the mechanism by which progesterone regulates the concentrations of endometrial oxytocin receptors is not known. From the observations of high concentrations of oxytocin receptors in chronically ovariectomized ewes (this study; Vallet *et al.* 1990; Lau *et al.*, 1992) and the rapid recovery of oxytocin receptors after the withdrawal of progesterone inhibition (this study), it is suggested that the gene coding for the synthesis of endometrial oxytocin receptors may be constantly 'switched on'. Progesterone would then inhibit the synthesis of endometrial oxytocin receptors. There is much evidence for the regulation of gene expression by steroids (see review by Rories & Spelburg, 1989). An alternative explanation is that progesterone may increase the degradation of oxytocin receptors. If the effect of progesterone is to regulate the synthesis of oxytocin receptors, then, in the absence of progesterone, the gene that codes for the synthesis of the receptors would be active and a large number of receptors would be synthesized. This would explain the findings of intrinsically high concentrations of endometrial oxytocin receptors in ovariectomized ewes (Vallet *et al.*, 1990; Lau *et al.*, 1992) and the rapid recovery of the concentrations of endometrial oxytocin receptors after the withdrawal of exogenous progesterone (this study). However, this suggestion does not exclude the possibility that other factors, not yet identified, may be involved in the regulation of oxytocin receptors in the uterus.

We thank R. C. Fry, T. L. Purdon and P. A. Weston for their technical support and G. Simpson, J. Zaina and P. Langdon for care of the animals, and the Australian Meat and Livestock Research and Development Corporation for financial support.

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Received 9 July 1991