Regulatory effect of steroid hormones and fetal tissues on expression of oxytocin receptor in the endometrium of late pregnant ewes

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Oxytocin receptors play an important role in the establishment of pregnancy and parturition in ruminants. Previous studies in cyclic and early pregnant ewes have indicated that receptor concentrations are regulated by steroid hormones and fetal secretory products. This study investigated the effect of oestradiol and progesterone, or co-culture with placenta or corpus luteum on oxytocin receptor expression. Endometrial explants from late pregnant ewes were cultured for up to 96 h in various treatment combinations. After culture, tissues were subjected to *in situ* hybridization and autoradiography with ^{125}I -labelled oxytocin receptor antagonist to localize and measure the expression of oxytocin receptor mRNA and protein. Results were quantified as absorbance units from autoradiographs. Oxytocin receptors were confined to the endometrial luminal epithelium and both mRNA and ^{125}I -labelled oxytocin receptor antagonist binding were upregulated spontaneously in basic serum-free medium. Upregulation occurred earlier in the presence of oestradiol ($0.1\ \mu\text{mol l}^{-1}$) but the final receptor concentration was similar to that found in the basic medium. Continuous progesterone treatment ($1\ \mu\text{mol l}^{-1}$) and co-culture with corpus luteum both delayed the increase in oxytocin receptor mRNA, but a short initial (4 h) period in progesterone-free basic medium resulted in loss of the inhibitory effect. Co-culture with placental tissues had no effect. In conclusion, oxytocin receptor expression in the luminal epithelium increased immediately on removal from the maternal environment. This occurred regardless of treatment and did not require the presence of steroid hormones, but could be accelerated or delayed by oestradiol and progesterone, respectively. There may be an additional inhibitory factor present in the corpus luteum.

Introduction

Inhibition of the development of oxytocin receptors plays an important role in the establishment of pregnancy (Flint *et al.*, 1994a; Wathes and Lamming, 1995), whereas induction of oxytocin receptors during labour facilitates delivery of the fetus (Fuchs *et al.*, 1981; Pimental *et al.*, 1989; Sugimoto *et al.*, 1997). The expression pattern of oxytocin receptors in the uterus of sheep and cows has been studied throughout the oestrous cycle (Fuchs *et al.*, 1990; Ayad *et al.*, 1991; Stevenson *et al.*, 1994) and at parturition (Fuchs *et al.*, 1992a,b; Wathes *et al.*, 1996a; Wu *et al.*, 1996). Studies *in vitro* and *in vivo* have led to the general belief that oxytocin receptor expression is regulated principally at the transcriptional level, and that oestrogen and progesterone are important positive and negative regulators, respectively (Soloff, 1985).

In early gestation in sheep, oxytocin receptor expression is also inhibited by interferon τ which is produced by the trophoblast between days 10 and 21 of pregnancy (Roberts *et al.*, 1992; Stewart *et al.*, 1992). Low expression of oxytocin

receptors appears in the ovine endometrial luminal epithelium after about 21 days of pregnancy, when interferon τ production ceases, and remains until parturition when further upregulation occurs (Wathes *et al.*, 1996a; Wu *et al.*, 1996). Although progesterone may be responsible for the suppression of oxytocin receptors throughout pregnancy, there is evidence to indicate that the fetus also has a local influence on oxytocin receptor expression (Cerbito *et al.*, 1997; Parry *et al.*, 1997). Unknown factors in addition to progesterone secreted from luteal tissues have also been implicated in inhibiting the expression of oxytocin receptors in the endometrium of cyclic sheep (Sheldrick *et al.*, 1993).

The aim of this study was to elucidate the possible roles of a variety of putative regulatory agents on the expression of oxytocin receptors in late pregnant sheep using endometrial explant cultures. The experiments were classified into two sections: (i) treatments with the steroid hormones oestradiol and progesterone in various concentrations and combinations; and (ii) co-culture of endometrium with fetal cotyledonary tissues, allantochorion and corpus luteum. The expression of oxytocin receptor mRNA and binding activity were analysed by *in situ* hybridization and autoradiography with ^{125}I -labelled oxytocin antagonist, respectively.

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Revised manuscript received 8 October 1998.

Materials and Methods

Reagents

Chemicals were purchased from Sigma Chemical Co. (Poole) or Merck/BDH (Poole) unless otherwise specified.

Tissue collection and culture

Clun Forest ewes were mated at natural oestrus and killed by an overdose of pentobarbitone sodium administered i.v. (Euthatal; Rhone Merieux, Tallaght, Dublin) at 130–138 days of gestation. The mean duration of gestation is 147 days. The entire uterus was collected immediately, opened in a laminar flow hood and the fetus removed. Pieces of intercotyledonary endometrium and placentome were dissected and frozen in isopentane immersed in liquid nitrogen. The samples were collected within 15 min after the animals were killed (time 0) and stored at -80°C .

The endometrium was separated from the fetal allantochorion by manual dissection. Strips of intercotyledonary endometrium were transferred to basic medium unless otherwise specified (see below). All the collected tissues were dissected into 1 mm^3 pieces using a mechanical tissue chopper within 30 min after the animals were killed (McIlwain Laboratory Engineering Ltd, Surrey). The endometrial pieces were blotted with a sterile lens cleaning tissue and weighed to provide 0.15 g per culture dish. The tissues were placed on a metal grid (stainless steel, $30\text{ mm} \times 30\text{ mm} \times 0.5\text{ mm}$) cushioned with a layer of lens cleaning tissue in dishes ($50\text{ mm} \times 15\text{ mm}$, single dent; BDH) containing 6 ml treatment medium (see below). In co-culture experiments the corpus luteum, pieces of allantochorion taken from the intercotyledonary regions and fetal cotyledon were also collected. The placentomes were separated manually by gentle squeezing into fetal and maternal components to obtain fetal cotyledon. The fetal side consisted of trophoctoderm with underlying stromal tissue of mesodermal origin, although some maternal epithelial syncytium was still attached. The fetal tissues were dissected into pieces as described earlier. For culture of these tissues, endometrium (0.1 g per dish) was spread out in the middle of the metal grid and surrounded by finely chopped corpus luteum, fetal cotyledonary tissues or strips of allantochorion (0.1 g per dish, respectively).

The tissues were cultured at 37°C with 5% CO_2 for up to 96 h in a humidified incubator. At the end of the culture period, endometrial explants were collected for subsequent analysis. All the tissue pieces from each grid were mounted in cryo-M-bed (Bright, Huntingdon), frozen in dry ice and stored at -80°C . The viability of the tissue after 96 h in culture was checked by examining the histology of the explants by haematoxylin and eosin staining.

Medium preparation

The basic medium used was Dulbecco's modified Eagle's medium/nutrient F12 mix (1:1) with 15 mmol Hepes l^{-1} (Gibco BRL, Paisley) containing penicillin sodium (50 iu ml^{-1} ;

NVS, Fenton, Stoke-on-Trent), streptomycin sulphate (50 mg ml^{-1} ; Sigma), 1.125 g sodium hydrocarbonate l^{-1} and bovine serum albumin (1.125 g l^{-1} ; Sigma). The medium was filtered (0.22 μm filter; Millipore, Watford). Finally, 1 ml insulin–transferrin–selenium mixture (ITS) was added to 1 l filtered medium to provide final concentrations of $5\text{ }\mu\text{g l}^{-1}$, $5\text{ }\mu\text{g l}^{-1}$ and 5 ng l^{-1} , respectively. The medium was stored at 4°C . Stock solutions of progesterone (3.18 mmol l^{-1}) and oestradiol (50 mmol l^{-1}) were prepared in absolute ethanol. These were then diluted to the working concentrations in basic medium through a serial dilution.

Experimental treatments

All treatments were tested on tissue collected from three separate ewes unless otherwise specified. Each treatment per animal was performed in triplicate.

Experimental group 1. This group included the following treatments: (i) control (basic medium); (ii) oestradiol ($0.1\text{ }\mu\text{mol l}^{-1}$ and $1\text{ }\mu\text{mol l}^{-1}$); (iii) discontinuous progesterone ($0.1\text{ }\mu\text{mol l}^{-1}$ and $1\text{ }\mu\text{mol l}^{-1}$); (iv) continuous progesterone ($1\text{ }\mu\text{mol l}^{-1}$); and (v) fetal calf serum (FCS, 5% v/v). The medium was changed every 24 h.

All treatments except the continuous progesterone treatment had an interval of 2–4 h between the removal of the endometrium from the maternal system and culture in the treated medium. During this interval, the endometrium was processed in basic medium. For the continuous progesterone treatment, endometrium was placed into medium containing progesterone immediately after removal from the animal and was maintained in this throughout processing. All samples treated with oestradiol were collected at 0, 12, 24 and 48 h; the rest of the samples were collected at 0, 48 and 96 h.

Experimental group 2. This group contained the following treatments: (i) control (basic medium); co-culture of endometrial explants with (ii) corpus luteum, (iii) allantochorion, and (iv) fetal cotyledonary tissues. The medium was changed every 24 h. The medium removed was collected and stored at -20°C . Tissue samples were collected at 0, 48 and 96 h.

Progesterone radioimmunoassay

The concentration of progesterone in the collected medium from the co-culture experiments was analysed by radioimmunoassay as described by Wathes and Porter (1982). The detection limit was $0.003\text{ }\mu\text{mol l}^{-1}$.

In situ hybridization

Sections (10 μm thick) of endometrial explants frozen in blocks of cryo-M-bed were cut and thaw-mounted on slides coated with poly-L-lysine (0.1 mg ml^{-1}). The sections were fixed in 4% (w/v) paraformaldehyde in PBS ($0.13\text{ mol NaCl l}^{-1}$,

0.007 mol Na_2HPO_4 l^{-1}) for 5 min, washed in PBS (3×2 min), and dehydrated in 75% and then 95% absolute ethanol for 5 min. The sections were stored in 95% absolute ethanol at 4°C .

The *in situ* hybridization procedures were applied as described by Wathes *et al.* (1996a). Briefly, the oxytocin receptor DNA probe (antisense; 45mer synthetic oligonucleotide), end labelled with [^{35}S]dATP (NEN Research Products, Stevenage; 100 000 c.p.m. per 100 μl hybridization buffer per slide), was added to the sections, which were then covered with a parafilm coverslip and incubated at 43°C overnight. After incubation, the sections were washed at room temperature for 30 min and placed in $1 \times$ sodium saline citrate (SSC; 15 mmol sodium chloride l^{-1} , 15 mmol sodium citrate l^{-1} , pH 7.0) containing 0.2% (w/v) sodium thiosulphate-5 hydrate for 1 h at 55°C . The slides were then dehydrated in a gradient of ethanol, air-dried and exposed to hyperfilm- β_{max} (Amersham International plc, Amersham) for 2 weeks. A labelled oligonucleotide of the sense sequence was used as the negative control for each sample. Sections from the uterus of a ewe in oestrus were used as the positive control in each batch of sections processed.

Autoradiography

The method was based on that described by Ayad *et al.* (1991). Sections (20 μm thick) were cut from frozen explant samples, pre-washed in ice-cold phosphate buffer (0.1 mol l^{-1} , pH 7.4, 0.1% (w/v) BSA) without magnesium chloride (MgCl_2) and incubated with 300 μl $\text{d}(\text{CH}_2)_5$ [$\text{Tyr}(\text{Me})^2\text{Thr}^4\text{TyrNH}_2$]-vasotocin (^{125}I -labelled oxytocin receptor antagonist; 1250 c.p.m. ml^{-1}) in buffer containing MgCl_2 (2.04 g l^{-1}). This gave the maximum binding. The nonspecific binding solution containing excess unlabelled oxytocin (10 mg ml^{-1} ; Bachem UK Ltd, Saffron Walden) was added to the control slides. The slides were then incubated at room temperature for 1 h. After incubation, the slides were washed vigorously in fresh ice-cold phosphate buffer containing MgCl_2 (3×1 min), washed for 1 min in ice-cold distilled water, and then air-dried at room temperature. The slides were then exposed to hyperfilm- β_{max} for 48 h.

Photographic emulsions

The procedures were similar to the instructions provided by the manufacturer (LM-1, Amersham International plc, Amersham). Dried slides were dipped vertically into the emulsion for 5 s at 43°C and allowed to dry horizontally for 10 min first at room temperature and then on a metal plate pre-cooled with dry ice. The slides were then placed into a light-tight box with anhydrous silica gel, sealed and incubated at 4°C for 3 weeks. After incubation, the slides were dipped into developer (Phenisol; Ilford Limited, Ilford) for 5 min, stop bath (0.5% (v/v) acetic acid) for 1 min, fixative (47% (w/v) sodium thiosulphate pentahydrate) for 10 min and then distilled water for at least 10 min before counterstaining with Harris' haematoxylin and eosin to identify the types of cell.

Data analysis

Images of the films from both the *in situ* hybridization and ^{125}I -labelled oxytocin receptor antagonist autoradiography experiments were quantified by measuring the absorbance of specific areas (identified using haematoxylin and eosin staining) using an image analysis system (Seescan plc, Cambridge). The results were expressed as arbitrary absorbance units on a linear scale from 0.01 to 2.1. Measurements were made of the sense and antisense images obtained from four sections per sample and three readings per section, giving a total of 12 readings per sample per time point. As oxytocin receptor expression was confined to the luminal epithelium, measurements were only taken from this type of cell, which was the only region to give a positive image on the film. Each area measured contained a minimum of 150 epithelial cells (see Fig. 1). The sense values were subtracted from the antisense values to produce a mean value of specific hybridization in each region for each sample. The inter-batch coefficient of variation (calculated from the control sections from a ewe in oestrus) was 12%. All the samples from the same tissue culture experiment were processed for *in situ* hybridization at the same time to remove any inter-batch variation in the analysis of treatment effects. The intra-batch coefficient of variation (calculated from duplicate sections of the same sample) was 18%.

Data from each treatment were analysed by a three-way ANOVA with the incubation time (h), the treatment effect (in comparison with the control at each time point) and the animal from which the tissue was obtained as factors. If the result from the initial ANOVA showed a significant treatment effect ($P < 0.05$), the data at each specific time point were subsequently analysed by Newman Keul's test to investigate the treatment effect. Similarly, the time effect was examined using the same method within the same treatment.

Results

Spontaneous increase in oxytocin receptor expression in endometrial explants

Data from all the experiments were pooled to define the changing pattern of oxytocin receptor expression over time in control tissues. Expression of oxytocin receptor mRNA in the unprocessed endometrium at 130–138 days of pregnancy was confined to the luminal epithelium and the concentration was 0.04 ± 0.007 (mean $\pm$ SEM absorbance units, $n = 6$ animals). The concentration of oxytocin receptor mRNA in chopped endometrial luminal epithelium collected at the start of culture (0 h) was 0.03 ± 0.006 absorbance units ($n = 12$ animals) and did not differ significantly from that in the untreated intact endometrial luminal epithelium. Oxytocin receptor mRNA started to increase spontaneously between 12 and 24 h of incubation and reached a peak at an absorbance of 0.21 ± 0.025 between 48 and 72 h (a significant increase, $P < 0.0001$; $n = 12$ animals), remaining at this concentration until 96 h (Figs 1 and 2). ^{125}I -labelled oxytocin receptor antagonist binding showed a similar trend, except that the receptor binding started to increase between 0 and 12 h, earlier than the upregulation of the mRNA.

Fig. 1. (a) Expression of oxytocin receptors in explant cultures of intercotyledonary endometrium obtained from a ewe on day 135 of pregnancy after 48 h of incubation and localized using *in situ* hybridization and (b) the corresponding sense control. (c) Oxytocin binding sites localized using autoradiography with ^{125}I -labelled oxytocin receptor antagonist and (d) nonspecific binding, containing excess unlabelled oxytocin peptide, used as the negative control. (e) Photographic emulsion showing that oxytocin receptor mRNA was expressed specifically in the endometrial luminal epithelium (LE) and (f) the corresponding sense control. Scale bars represent (a–d) 3 mm (e,f) 10 μm .

Experimental group 1: hormone treatments

Oestradiol ($0.1 \mu\text{mol l}^{-1}$) upregulated the concentration of oxytocin receptor mRNA significantly after 12 h of incubation compared with the corresponding controls. There was no significant effect at 24 and 48 h, and the final mRNA concentration was similar to that found in the controls (Table 1). Oestradiol ($0.1 \mu\text{mol l}^{-1}$) also significantly increased the binding of ^{125}I -labelled oxytocin receptor antagonist after 12 h of culture. This increase was no longer significant at 24 h and had disappeared by 48 h (Table 2). Oestradiol at a

higher concentration ($1 \mu\text{mol l}^{-1}$) had no effect on oxytocin receptor mRNA expression.

Discontinuous progesterone treatment at both 0.1 and $1.0 \mu\text{mol l}^{-1}$ (present from when the processed tissue was placed on the grids, approximately 2–4 h after removal from the animal) had no significant effect on the spontaneous increase in the concentration of oxytocin receptor mRNA at either 48 or 96 h. The continuous progesterone treatment ($1 \mu\text{mol l}^{-1}$, present from immediately after tissue removal from the animal) inhibited oxytocin receptor expression at 48 h of incubation, but this inhibitory effect disappeared by 96 h of

Fig. 2. The concentration of (a) oxytocin receptor mRNA and (b) oxytocin receptor binding localized in the luminal epithelium using *in situ* hybridization and autoradiography with ^{125}I -labelled oxytocin receptor antagonist. The results are expressed as absorbance units (mean $\pm$ SEM, $n = 12$ animals). Samples were collected at regular intervals from the start of culture (0 h). A significant and spontaneous upregulation of oxytocin receptor mRNA was observed between 12 and 48 h. The oxytocin receptor mRNA concentration reached peak values between 48 and 72 h, and remained high until 96 h. The receptor binding activity increased at a constant rate from 0 h, peaked at 48 h, and remained high at 96 h. Significant differences are indicated: $b > a$, $P < 0.01$; $c > b$, $P < 0.01$.

incubation (Table 1). In contrast, neither progesterone treatment showed any effect on the binding of ^{125}I -labelled oxytocin receptor antagonist at either time point (Table 2).

There was no significant effect of 5% FCS on either the timing of the spontaneous increase in expression of oxytocin receptor mRNA or in the final concentration reached (Table 1).

Experimental group 2: co-culture experiments

The co-culture of endometrium with corpus luteum significantly inhibited expression of oxytocin receptor mRNA in the endometrial luminal epithelium after 48 h of incubation, but the difference was no longer significant by 96 h (Table 3). Co-culture of fetal cotyledonary tissues and allantochorion with maternal endometrium did not have any significant effect on the expression of oxytocin receptor mRNA at either 48 or 96 h. Neither of the co-culture treatments had any significant effect on the binding of ^{125}I -labelled oxytocin receptor antagonist (Table 3).

The corpus luteum explants secreted significant concentrations of progesterone into the medium at both 48 and 96 h (0.6 ± 0.21 and $0.2 \pm 0.1 \mu\text{mol l}^{-1}$, respectively). There were low but detectable concentrations of progesterone in the medium collected from the co-cultures with fetal cotyledon (0.003 ± 0.0012 and $0.001 \pm 0.0004 \mu\text{mol l}^{-1}$ at 48 and 96 h, respectively), whereas cultures containing only endometrium produced no detectable progesterone at either time point (concentration in medium $< 0.0003 \mu\text{mol l}^{-1}$).

Discussion

The experiments reported here were designed to provide further information on the effect of steroid hormones and the involvement of tissue-specific factors on the expression of endometrial oxytocin receptors in late pregnancy. The advantage of using explant culture over cell culture is that any regulatory effect from endometrial stromal cells is preserved. This provides a better system to evaluate the requirement of local regulatory factors and hence a more accurate observation on the regulation of expression of oxytocin receptors. The combined use of *in situ* hybridization and ^{125}I -labelled oxytocin receptor antagonist autoradiography on the same samples allowed a direct comparison to be made between regulation of expression of oxytocin receptors at the transcriptional and post-transcriptional levels, and determination of the cellular localization. This study shows that in pregnant endometrium, expression of oxytocin receptors is confined to the epithelium.

The endometrial explants used in this study were also cultured in fully defined serum-free medium to elucidate the effect of the individual treatments. However, results from the treatment group in which FCS was included were similar to those found in the serum-free controls with respect to mRNA expression. This finding, coupled with observations of tissue morphology, confirmed that the endometrial explants could survive in serum-free medium for at least 96 h. Furthermore, the concentration of oxytocin receptor mRNA and ^{125}I -labelled oxytocin receptor antagonist binding measured at 48 and 96 h in the explants were comparable to those reported in endometrial epithelium from ewes undergoing spontaneous labour (Wathes *et al.*, 1996a).

The present study and other reports on endometrial explants of nonpregnant sheep (Sheldrick *et al.*, 1993) and endometrial epithelial cultures of cows (Horn *et al.*, in press) show that endometrial oxytocin receptor concentrations increase spontaneously in culture. It is unclear what causes this upregulation. It is possible that maximal expression of oxytocin receptors may require a specific factor that is either inactivated or suppressed for most of the time within the normal uterine environment. This factor must be produced by the endometrium of both pregnant and nonpregnant animals and is unlikely to be oestradiol for the reasons discussed below. Alternatively, removal of the endometrium from the maternal system may introduce stress into the explant tissues such as cell death, changes in osmolality and temperature shock during the processing procedures. Signals such as these may trigger an acute response (Dudley *et al.*, 1993) which subsequently influences expression of the oxytocin receptor.

Table 1. Effect of hormone treatment on expression of oxytocin receptor mRNA in the luminal epithelium of endometrial explants from late pregnant ewes

Treatment	Time in culture			
	12 h	24 h	48 h	96 h
Control	0.03 ± 0.014 ^a	0.07 ± 0.029	0.19 ± 0.061	nt
Oestradiol (0.1 µmol l ⁻¹)	0.06 ± 0.024 ^b	0.10 ± 0.028	0.16 ± 0.047	nt
Oestradiol (1.0 µmol l ⁻¹)	0.03 ± 0.019	0.07 ± 0.029	0.25 ± 0.033	nt
Control	nt	nt	0.22 ± 0.021 ^c	0.21 ± 0.026
Continuous progesterone (1.0 µmol l ⁻¹)	nt	nt	0.12 ± 0.040 ^d	0.21 ± 0.070
Discontinuous progesterone (1.0 µmol l ⁻¹)	nt	nt	0.18 ± 0.052	0.25 ± 0.100
Discontinuous progesterone (0.1 µmol l ⁻¹)	nt	nt	0.20 ± 0.032	nt
Control	nt	nt	0.18 ± 0.061	0.15 ± 0.033
Fetal calf serum (5% v/v)	nt	nt	0.20 ± 0.038	0.15 ± 0.031

Each treatment was tested in triplicate on tissues from three ewes. Results are expressed as absorbance units (mean ± SEM) and are analysed in comparison with tissues from the corresponding controls from the same time point.

Discontinuous progesterone treatment was in basic control medium for approximately 4 h during tissue processing, whereas continuous progesterone treatment was processed in medium containing progesterone.

Data were analysed by ANOVA with time, treatment and animal as factors. Significant treatment effects at a particular time point are shown: b > a and d < c; *P* < 0.01. nt, not tested.

Table 2. Effect of steroid treatment on ¹²⁵I-labelled oxytocin receptor antagonist binding in the luminal epithelium of endometrial explants from late pregnant ewes

Treatment	Time in culture			
	12 h	24 h	48 h	96 h
Control	0.03 ± 0.010 ^a	0.04 ± 0.014	0.09 ± 0.020	nt
Oestradiol (0.1 µmol l ⁻¹)	0.06 ± 0.012 ^b	0.07 ± 0.014	0.10 ± 0.033	nt
Control	nt	nt	0.06 ± 0.012	0.10 ± 0.024
Continuous progesterone (1.0 µmol l ⁻¹)	nt	nt	0.06 ± 0.024	0.08 ± 0.007
Discontinuous progesterone (1.0 µmol l ⁻¹)	nt	nt	0.05 ± 0.024	0.10 ± 0.014

Each treatment was tested in triplicate on tissues from three ewes. Results are expressed as absorbance units (mean ± SEM) and are analysed in comparison with tissues from the corresponding controls from the same time point.

Discontinuous progesterone treatment was in basic control medium for approximately 4 h during tissue processing, whereas continuous progesterone treatment was processed in medium containing progesterone.

Significant treatment effects at a particular time point are shown: b > a; *P* < 0.01. nt, not tested.

Table 3. Effect of co-culture on expression of oxytocin receptor mRNA and ¹²⁵I-labelled oxytocin receptor antagonist binding in the luminal epithelium of endometrial explants from late pregnant ewes

Treatment	Time in culture			
	48 h	96 h	48 h	96 h
	Oxytocin receptor mRNA		¹²⁵ I-labelled oxytocin receptor antagonist binding	
Control	0.20 ± 0.005 ^a	0.19 ± 0.102	0.07 ± 0.002	0.08 ± 0.023
Corpus luteum	0.10 ± 0.005 ^b	0.15 ± 0.021	0.05 ± 0.002	0.06 ± 0.033
Control	0.11 ± 0.038	0.10 ± 0.029	0.07 ± 0.010	0.07 ± 0.043
Fetal cotyledon	0.12 ± 0.029	0.11 ± 0.026	0.04 ± 0.010	0.06 ± 0.014
Allantochoion	0.14 ± 0.023	0.10 ± 0.026	0.06 ± 0.012	0.05 ± 0.038

Each treatment was tested in triplicate on tissues from three ewes. Results are expressed as absorbance units (mean ± SEM) and are analysed in comparison with tissues from the corresponding controls from the same time point.

Significant treatment effects at a particular time point are shown: b < a; *P* < 0.01.

Many studies *in vivo* have shown the positive effect of oestradiol on expression of oxytocin receptors. However, a number of reports indicate that the action of oestradiol can be biphasic, both inducing and inhibiting the expression of oxytocin receptors depending on the concentration and duration of treatment (Vallet *et al.*, 1990; Larcher *et al.*, 1995; Wathes *et al.*, 1996b). The oxytocin receptor gene promoter contains three half-palindromic oestrogen responsive elements (Inoue *et al.*, 1994), indicating that oestrogen may regulate the expression of the oxytocin receptor directly. However, in the present study, oestradiol was not required for the upregulation of oxytocin receptor mRNA or binding in the luminal epithelial cells and there was no significant difference between the control and oestradiol treatments at 48 h. This finding is consistent with studies *in vitro* showing that binding of tritiated oxytocin to the endometrium increases significantly and spontaneously up to 96 h in culture. Oestradiol either had no effect ($< 1 \mu\text{mol l}^{-1}$) or inhibited the binding activity at a high concentration ($100 \mu\text{mol l}^{-1}$) in ovine endometrial explants and bovine epithelial cell cultures after 96 h of incubation (Sheldrick and Flick-Smith, 1993; Horn *et al.*, in press). Therefore, there is a discrepancy between the action of oestradiol *in vivo* and *in vitro*. In the present study, the effect of oestradiol was examined at an earlier series of time points from 12 h after the start of culture. This approach was chosen to determine whether oestrogen influences the rate or timing of the spontaneous increase, without necessarily causing a greater final concentration. The results of this study are in agreement with previous reports that there was no effect at 48 h. However, oestradiol did stimulate the increase in expression of oxytocin receptor mRNA to occur slightly earlier during the culture. This finding indicates that oestradiol either enhances the efficiency of the transcriptional process or potentiates the half-life of oxytocin receptor mRNA such that a greater amount accumulates. It has been suggested that oestradiol prolongs the poly(A)-tail of the oxytocin receptor mRNA to stabilize the transcript in the kidney (Breton *et al.*, 1996). Oestradiol also stimulated the binding of ^{125}I -labelled oxytocin receptor antagonist significantly after 12 h of incubation, indicating that the effect of oestradiol on oxytocin receptor expression is at the transcriptional level.

Progesterone can inhibit the expression of oxytocin receptors in steroid-treated ovariectomized sheep *in vivo*, but the inhibitory effect on the luminal epithelial cells cannot be maintained for longer than about 12–14 days (Vallet *et al.*, 1990; Wathes *et al.*, 1996b). In the present study, progesterone did not significantly inhibit the expression of oxytocin receptors *in vitro* if the endometrium was temporarily exposed to a progesterone-free environment for about 4 h. In contrast, continuous progesterone treatment significantly inhibited the upregulation of the oxytocin receptor mRNA at 48 h, but the effect could not be sustained for 96 h. This indicates that progesterone inhibits oxytocin receptor expression indirectly. Unlike oestradiol, the binding activity of the ^{125}I -labelled oxytocin receptor antagonist was not significantly different from the control in both progesterone treatments during the period studied, although transcription of the oxytocin receptor gene was inhibited in the continuous progesterone treatment at 48 h. This result implies that

progesterone regulates oxytocin receptor expression at the transcriptional level. However, post-transcriptional control of oxytocin receptor expression may not be influenced by progesterone.

These data are consistent with various *in vitro* experiments indicating that a continuous progesterone environment is essential (even if not sufficient on its own) to keep oxytocin receptor expression at a basal level. Administration of RU486 (a progesterone antagonist) or epostane (a progesterone synthase inhibitor) initiates parturition in late pregnant cows or sheep (Ledger *et al.*, 1985; Li *et al.*, 1991) and similar experiments with RU486 in rats showed that treatment resulted in an increase in uterine oxytocin receptor expression (Fang *et al.*, 1996, 1997). In the present study, disruption of the progesterone-dominated environment for as little as 4 h was sufficient to remove the inhibitory effect of progesterone. This result also favours the proposed mechanism that once the stimulatory factors are switched on, they either cannot be switched off or antagonize the inhibitory effect of progesterone.

Co-culture of endometrium with corpus luteum inhibited the spontaneous upregulation of oxytocin receptors in the endometrial luminal epithelium. This is consistent with previous findings in nonpregnant sheep (Sheldrick *et al.*, 1993). This implies that the inhibitor of oxytocin receptor expression secreted from the cyclic corpus luteum remains active during late pregnancy. Although the inhibitory pattern was similar to that of the continuous progesterone treatment described earlier, the inhibitory effect of the corpus luteum remained even when the endometrium was exposed to a progesterone-free environment for 4 h, whereas the discontinuous progesterone treatment failed to suppress oxytocin receptor expression. The progesterone concentration tested ($1 \mu\text{mol l}^{-1}$) was in the same concentration range as the progesterone output by the luteal co-cultures ($0.6 \mu\text{mol l}^{-1}$). Therefore, the unknown factor is unlikely to be progesterone. Although such a factor could modulate oxytocin receptor expression, it may not play an essential role because ovariectomy of sheep in midpregnancy does not significantly affect pregnancy outcome (Liggins and Thorburn, 1994).

Interferon τ produced by the trophoblast in early pregnancy in sheep strongly inhibits the expression of oxytocin receptors (Stewart *et al.*, 1992), which is an essential step to the establishment of pregnancy. The trophoblast stops producing interferon τ at about 3 weeks after mating (Flint *et al.*, 1994b). Although oxytocin receptor expression in the endometrium returns after the disappearance of interferon τ on about day 22 of pregnancy, its concentration remains low throughout gestation (Wathes *et al.*, 1996a). In the present study, co-culture of endometrium with fetal placental tissues or allantochorion did not have any regulatory effect on oxytocin receptor expression. Therefore, the suppression of oxytocin receptor mRNA during pregnancy is unlikely to be controlled directly by the placental tissues after the critical period that is necessary for the maternal recognition of pregnancy.

In summary, oestradiol facilitates the expression of oxytocin receptors in late pregnant endometrium, probably via a transcriptional mechanism. However, it is not essential for the full upregulation of oxytocin receptor transcription in

the epithelium. The spontaneous increase in oxytocin receptor expression in culture probably involves removal of an unknown specific regulator, which may be controlled by progesterone at the transcriptional, but not at the post-transcriptional level. An environment dominated continuously by progesterone is required to suppress the upregulation of oxytocin receptors. The withdrawal of progesterone for a brief period is sufficient to switch on oxytocin receptor expression, and the inhibitory effect of progesterone is subsequently irrecoverable. This mechanism is not influenced directly by fetal placental tissues, but it may be modulated by specific factors from the corpus luteum.

The authors would like to thank John Thompson and David Manners for care of the animals, and the Royal Veterinary College for financial support. The oxytocin receptor antagonist was kindly supplied by M. Manning.

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