Changes in progesterone and oestrogen receptor mRNA and protein during maternal recognition of pregnancy and luteolysis in ewes

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ABSTRACT

This study characterized changes in levels of mRNA and protein for endometrial oestrogen receptors (ERs) and progesterone receptors (PRs) during luteolysis and maternal recognition of pregnancy. For cyclic and pregnant ewes, endometrium was collected on days 10, 12, 14, or 16 post-oestrus (4 ewes/day for each status) for the measurement of ER and PR mRNA and protein. The amount of receptor mRNA is expressed in relative units above background, measured from radiographs of dot-blot hybridization of total endometrial RNA with ER and PR cDNAs. At hysterectomy, jugular vein blood samples were collected and assayed for progesterone, total corpus luteum weight was recorded and, in vitro, endometrial oxytocin-stimulated inositol phosphate formation was estimated. In pregnant ewes, plasma progesterone increased gradually between days 10 and 16 (P<0·01), corpus luteum weight was stable at approximately 0·8 g and oxytocin did not stimulate endometrial formation of inositol phosphates in vitro. In contrast, in cyclic ewes, plasma progesterone decreased from day 10 to day 16 (P<0·01), corpus luteum weight decreased after day 14 to approximately 0·48 g (P=0·05) and oxytocin stimulated an increase of approximately 1300% in the endometrial formation of inositol phosphates on day 16. cDNAs specifically hybridized with 1·6 and 3·1 kb transcripts for PR mRNA and a 6·5 kb transcript for ER mRNA. In cyclic ewes, the amount of PR mRNA increased from day 10 to maximum levels on days 14–16. The number of PRs decreased from day 10 (2·25 pmol/mg DNA) to day 12 (0·98 pmol/mg DNA) and then increased from day 14 to day 16 (2·8 pmol/mg DNA). In pregnant ewes, PR mRNA levels were greatest on days 10–12 and decreased by approximately 50% by day 16. In contrast, the number of PRs was relatively unchanged from day 10 to day 16 (1·53 to 1·03 pmol/mg DNA). In cyclic ewes, the amount of ER mRNA was lowest at day 10 and increased fivefold by day 16. The number of ERs remained relatively unchanged from day 10 to day 14 (6·07 pmol/mg DNA) and increased by day 16 (16·12 pmol/mg DNA). In pregnant ewes, ER mRNA decreased by approximately 80% from day 12 to day 16. Similarly, the number of ERs decreased from day 10 to day 16 (5·41 to 2·05 pmol/mg DNA). Correlations between ER mRNA and PR mRNA (r=0·68), ERs and PRs (r=0·50) and ER mRNA and ERs (r=0·50) were high (P<0·01). PR mRNA and PRs, PR mRNA and ERs, and ER mRNA and PRs were not correlated (P>0·1). Pregnancy had the apparent effect of stabilizing the number of endometrial PRs and inhibiting ER production by decreasing both the amount of ER mRNA and ER protein.

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INTRODUCTION

The establishment of pregnancy in domestic ruminants requires the maintenance of an ‘embryotrophic’ uterine environment and the abrogation of the luteolytic pulses of prostaglandin F2α which are responsible for the demise of the corpus luteum (CL) and the return to oestrus (Bazer et al. 1991). Products of the developing conceptus (i.e. ovine and bovine type-I trophoblast interferons) have been implicated...
in the latter mechanism by inhibiting the rapid increase in oxytocin receptors on the uterine endometrium which occurs just prior to luteolysis (days 13–14; Bazer et al. 1991). Interactions between secretory products of the developing conceptus and the steroid hormones, oestrogen and progesterone, which result in extended maintenance of a progestational endometrium, are less well defined.

Uterine histotrophs contain numerous steroid-regulated secretory products which are thought to be essential for conceptus growth and development (Miller et al. 1977a; Bazer & Roberts, 1983; Vallet et al. 1987; Bazer et al. 1991; Brigstock, 1991; Ko et al. 1991). Since chronically elevated progesterone is known to down-regulate the progesterone receptor (Milgrom et al. 1972; Leavitt et al. 1974), secretory products of the conceptus must be involved in either maintaining or mimicking the actions of progesterone on the uterine endometrium. McCracken et al. (1984) emphasized this concept in his theory of the role of the conceptus in extending the 'progesterone block' to luteolysis.

Cyclic variations in endometrial steroid receptor concentrations have been described in cattle (Senior, 1975; Zelinski et al. 1982; Meyer et al. 1988), sheep (Koligian & Stormshak, 1977; Miller et al. 1977b; Findlay et al. 1982; Cherny et al. 1991) and pigs (Deaver & Guthrie, 1980). Particularly well-characterized is the phenomenon of oestrogen-induced synthesis of both oestrogen and progesterone receptors (Aronica & Katzenellenbogen, 1991), with concentrations of both receptors being greatest around oestrus in ewes (Koligian & Stormshak, 1977; Miller et al. 1977b; Cherny et al. 1991). Progesterone inhibits the synthesis of its own receptor (Leavitt et al. 1974; Vu Hai et al. 1977), as well as the oestrogen receptor (Hsu et al. 1976; Miller & Moore, 1976; Koligian & Stormshak, 1977; Read et al. 1988). However, recent comparative investigations are making it clear that progesterone affects progesterone receptor dynamics differently in progestational tissue. For example, West et al. (1986) found progesterone to up-regulate the progesterone receptor in endometrium and, yet, to down-regulate the progesterone receptor in adjacent myometrium and cervix. Progesterone receptors are also maintained by progesterone in the decidua basalis during mid-pregnancy in ovariectomized rats without a requirement for oestrogen (Ogle et al. 1990). Maslar & Lazar (1989) have made similar observations in primary cultures of macaque decidual cells wherein incubation with progesterone, but not oestrogen, maintained progesterone receptors. These diverse mechanisms of progesterone receptor regulation are entirely consistent with the essential role of oestrogen and progesterone in maintaining reproductive cycles and pregnancy. Steroid receptor dynamics in the endometrium of the pregnant ewe also appear to be significantly altered, regulated in part by secretory products from the conceptus (Warren et al. 1973; Koligian & Stormshak, 1977; Cherny et al. 1991). The nature of these interactions has yet to be clearly defined, but theoretically they could involve the regulation of receptor gene transcription, translation, or degradation and recycling of receptor proteins. Thus, to address this question, we have made simultaneous measurements of changes in endometrial oestrogen and progesterone receptor proteins and their mRNAs during luteolysis in ewes and during the corresponding period of pregnancy when signals from the conceptus prevent luteal regression (days 10–16).

MATERIALS AND METHODS

Animals

Mature crossbred Rambouillet ewes were checked daily for oestrous behaviour using vasectomized rams. Ewes exhibiting at least two oestrous cycles of normal duration (i.e. 16–18 days) were assigned at random to either cyclic or pregnant status. Ewes assigned to the pregnant status were mated to an intact ram on the morning of the onset of oestrus (day 0), and 12 and 24 h later. Ewes were hysterectomized 10, 12, 14, or 16 days post-oestrus (n = 4 ewes/day in each status). At hysterectomy, a jugular vein blood sample was collected to measure plasma progesterone and CL were excised and weighed. Endometrium was collected, snap-frozen in liquid nitrogen and stored at −80 °C until assayed for oestrogen and progesterone receptor mRNA and protein. Care was taken to include equal amounts of caruncular and intercaruncular endometrium from both uterine horns in assays for progesterone and oestrogen receptor protein and mRNA. For the inositol phosphate assay, endometrium from the uterine horn ipsilateral to the CL was collected into ice-cold Krebs–Ringer bicarbonate buffer (KRB) containing 10 mM glucose and 10 μM myo-inositol (Mirando et al. 1990b).

Progesterone radioimmunoassay (RIA)

The concentration of progesterone in 200 μl plasma was determined by RIA (Mirando et al. 1990b). The average extraction efficiency was 84.8%, the assay sensitivity was 10 pg/tube and the intra- and inter-assay coefficients of variation were 6.2 and 9.5% respectively.
Inositol phosphate metabolism

For the determination of inositol phosphate metabolism, endometrium in ice-cold KRB was prepared and incubated as previously described in detail (Mirando et al. 1990a,b). Approximately equal amounts of caruncular and intercaruncular endometrium (100 mg total) were incubated in KRB for 2 h with 10 μCi [3H]inositol (specific activity 19 Ci/mmol; Amersham, Arlington Heights, IL, U.S.A.) and for 30 min in the presence of 0 or 100 mM oxytocin (Sigma Chemical Co., St Louis, MO, U.S.A.). Incubations were terminated by replacing KRB with 1 ml ice-cold trichloroacetic acid which was then removed by extracting five times with 5 ml diethyl ether. The incorporation of [3H]inositol into inositol phosphates was determined after anion-exchange chromatography and liquid scintillation spectrometry as previously described in detail (Mirando et al. 1990a,b).

RNA extraction

RNA was isolated using the acid guanidinium thiocyanate–phenol–chloroform extraction modification of the single-step method described by Puissant & Houdebire (1990). Briefly, 1 g frozen endometrium was homogenized at 4°C in 10 ml 4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7, 0·5% sarkosyl and 0·1 M mercaptoethanol, with three to four bursts of 5 s with a Tissuemizer (Tekmar Co., Cincinnati, OH, U.S.A.). Homogenization was followed by successive additions of 1 ml 2 M sodium acetate, pH 4, and 10 ml water-saturated phenol followed by gentle mixing and 2 ml chloroform followed by vortexing for 15 s. The homogenate was centrifuged at 5000 × g for 20 min and the upper phase was recovered and precipitated with 10 ml isopropanol at −80°C for 1 h. The precipitated RNA was then pelleted by centrifugation at 5000 × g for 10 min. Polysaccharides were solubilized by vigorous vortexing in 2 ml 4 M LiCl and the RNA was pelleted by centrifugation at 5000 × g for 15 min. The resulting pellet was dissolved in 2 ml 10 mM Tris, pH 7·5, 1 mM EDTA, 0·5% sodium dodecyl sulphate (SDS). Chloroform (2 ml) was added, followed by vortexing to mix the phases and centrifugation at 5000 × g for 10 min. The upper phase was collected, acidified to pH 5·0 with sodium acetate, precipitated with 2 ml isopropanol and stored overnight at −80°C. Insoluble material, which should have been pure RNA, was pelleted by centrifugation at 5000 × g for 30 min. RNA was washed with 1 ml ethanol, pelleted by centrifugation at 5000 × g for 10 min and then resuspended in 200 μl sterile distilled water. Absorbances at 260 and 280 nm were measured for a 1:200 dilution of this preparation and the ratio of absorbances (260:280) was calculated.

Hybridization

Total RNA from each ewe (10 and 20 μg) was loaded on triplicate 0·45 μm Nytran membranes using a microparticle filtration unit (Scheicher and Schuell, Keene, NH, U.S.A.). Yeast RNA (30 μg) was used as a background control. Blots were heated for 2 h in an oven at 80°C, washed in sterile water and prehybridized overnight at 42°C in 5 × SSC (1 × SSC is 15 mM NaCl, 15 mM sodium citrate), 5 × Denhardt’s, 50 mM NaPO4 (pH 6·5), 50% formamide, 0·1% SDS and 100 μg yeast RNA/ml. Hybridization was performed at 42°C for 16 h in the same buffer, except with 1 × Denhardt’s, containing the gel-purified cDNA insert which was randomly labelled (Prime-a-gene; Promega Corp., Madison, WI, U.S.A.) with [32P]dCTP (specific activity 3000 Ci/mmol; ICN Biochemicals Inc., Irvine, CA, U.S.A.) to a specific activity of approximately 106 c.p.m./μg DNA. Probes (cDNA) for oestrogen (OR8; Greene et al. 1986) and progesterone (Sp79; Conneeley et al. 1986) receptor mRNAs were generously provided by Dr B. W. O’Malley (Department of Cell Biology, Baylor College of Medicine, Houston, TX, U.S.A.). Following hybridization, filters were washed in 2 × SSC containing 0·1% SDS at room temperature for 30 min followed by 0·1 × SSC (0·1% SDS) for 30 min at 42°C. Hybridization signals were detected by exposing filters to XAR film (Eastman Kodak, Rochester, NY, U.S.A.) for 36 h at −70°C. Radiograms of dot blots were quantified using an EC910 densitometer (E-C Apparatus Corp., St Petersburg, FL, U.S.A.). Relative hybridization signal intensities are expressed as unit values above background, corrected for variations in loading.

Northern analysis of transcript size

To estimate the sizes of the transcripts for oestrogen and progesterone receptor mRNAs, poly(A)+ RNA was isolated, using a PolyAtract mRNA isolation kit (Promega Corp.), from total endometrial RNA from one cyclic and one pregnant ewe on day 16. After denaturation in loading buffer (24 mM Hepes, 6 mM sodium acetate, 1·2 mM EDTA, 50% deionized formamide, and 2·2 mM formaldehyde) for 15 min at 65°C followed by cooling on ice for 15 min, poly(A)+ RNA (25 μg) was electrophoresed overnight through a 1·5% agarose–formaldehyde gel (35 V) and transferred to a 0·45 μm nitrocellulose
membrane (Schleicher and Schuell) by capillary blotting. Blots were baked in an oven at 80°C for 2 h then prehybridized and hybridized as described above. Hybridization signal intensities were detected as described above and transcript sizes estimated (based on a 3700 base size for 28S and a 1900 base size for 18S rRNA) and compared with published reports.

**Steroid receptor determinations**

Uterine endometrial tissues were mechanically homogenized after rapid thawing by three 10-s bursts with a Tissumizer followed by 30-s periods of cooling. Ice-cold conditions were maintained throughout subsequent procedures unless otherwise noted. The exchange assay was performed by incubating receptor preparations with incubation buffer containing six concentrations of 3H-labelled ligand (1–17 nM progesterone and 0–3–3.0 nM oestradiol; Dupont NEN Research, Boston, MA, U.S.A.). A companion set of assay tubes containing a 100-fold molar excess of unlabelled ligand in addition to labelled ligand was used to determine non-specific binding. All receptor measurements were performed under conditions of endogenous steroid exchange. Sample radioactivity was measured in a Beckman LS-1800 liquid scintillation counter (Beckman Instruments, Palo Alto, CA, U.S.A.).

**Oestrogen receptor assay**

Endometrial tissues were homogenized in TEDSL buffer (5 ml/g) containing 0.05 M Tris, pH 7.8 at 4°C, 1.5 mM EDTA, 0.5 mM dithiothreitol, 0.25 M sucrose and 0.2 M leupeptin. Nuclear-myofibrillar pellets were obtained by centrifugation at 800 g for 20 min. The supernatant was recentrifuged for 1 h at 105 000 g, yielding the cytosolic fraction. The nuclear-myofibrillar pellet was washed three times by resuspension in TMDSL buffer (containing 2.5 mM MgCl₂ instead of EDTA), followed by centrifugation at 800 g for 20 min after each wash, and finally rehomogenized in TEDSL buffer using a Dounce homogenizer. A 0.25 ml aliquot of each preparation (0.05–0.15 mg DNA) was added to each assay tube containing TEDSL and ligand in a final volume of 0.3 ml. A 0.1 ml aliquot of the cytosolic fraction was incubated in a final volume of 0.3 ml in TEDSL buffer. Exchange was carried out at 22°C for 2 h, then at 4°C for 18 h. Steroid bound to the cytosolic receptor was separated from free steroid by dextran-coated charcoal. Free steroid was removed from the nuclear-myofibrillar pellet by three rinses with Tris–sucrose buffer, and finally suspended in 1 ml ethanol warmed to 30°C for 1 h. Particulates were pelleted after centrifugation at 1600 g for 10 min and the supernatant was decanted into a scintillation vial for counting.

**Progesterone receptor assay**

Endometrial tissues were homogenized at 0°C in fresh TGDL buffer (0.01 M Tris, pH 7.8 at 0°C, 30% glycerol, 1 mM dithiothreitol and 0.2 mM leupeptin). The homogenate was strained through two layers of gauze and centrifuged at 800 g for 15 min. The pellet contained nuclei and the supernatant was centrifuged again for 40 min at 157 000 g to yield the cytosolic fraction. The cytosolic receptor was partially purified by differential precipitation with ammonium sulphate (Ogle, 1981), and 100 μl of the partially purified cytosolic receptor were incubated with 0.2 ml TDGL at 0–4°C for 20–22 h. Bound steroid was separated from free steroid by use of dextran-coated charcoal.

The nuclear pellet was rinsed twice with fresh TDGL, suspended in TDGL buffer, and 0.1 ml aliquots containing 0.1–0.2 mg DNA were incubated for 22 h at 0–4°C. Free steroid was removed from the nuclear pellet by repeated rinsing with TDGL. After the final rinse, the nuclear pellet was resuspended in 0.6 ml buffer and poured into a scintillation vial (Ogle, 1986).

The term ‘cytosol receptors’ is used as an operational definition to refer to forms of oestrogen and progesterone receptors located in the soluble fraction after ultracentrifugation. It is generally believed that the cytosolic component arises from the extraction of unbound or loosely bound receptors from the nucleus during tissue manipulation and disruption in vitro. Gasc et al. (1984), King & Greene (1984) and Welshons et al. (1985) have shown by immunocytochemical localization and physical cell nucleation experiments that the receptors reside within the nucleus in situ.

**Protein and DNA determinations**

Protein concentrations in homogenates were assayed by the method of Lowry et al. (1951), and DNA levels were determined using a fluorometric procedure (Hill & Whatley, 1975).

**Expression of data and statistical analyses**

The amounts of oestrogen and progesterone receptor mRNAs, measured from dot blots, are expressed in units above background, corrected for loading variation. Data are least squares
means ± S.E.M. for three to four animals per status for each day of sampling. For oestrogen receptor and progesterone receptor assays, each value was corrected for non-specific binding. Binding levels were determined under equilibrium conditions from the analysis of a six-point saturation assay by computerized non-linear and linear regression techniques as described previously (Ogle et al. 1989). Average $K_d$ values for all assays were $0.9 ± 0.2$ nM and $2.3 ± 0.2$ nM for oestrogen and progesterone receptors respectively. Measurements of receptor binding activity are normalized for DNA. Total receptor content was determined for each endometrial sample as the sum of cytosol and nuclear values. All values are reported as least squares means ± S.E.M. for three to four animals per treatment group. Comparisons among least squares means were made using the general linear models procedures of the Statistical Analysis System (SAS Institute, 1985). For analysis of CL weight, CL number was included in the model as a covariate. For plasma progesterone concentrations, oestrogen receptor and progesterone receptor mRNAs and proteins, between-status comparisons were accomplished using regression analyses. Within-status comparisons among days were made using orthogonal contrasts. Simple and partial correlation coefficients, with and without the data adjusted for the effects of status, day and their interaction, were estimated using the correlation and manova procedures of the Statistical Analysis System (SAS Institute, 1985).

RESULTS

Corpus luteum weights (Fig. 1) and plasma progesterone concentrations (Fig. 2) were measured from samples taken at the time of surgery to determine the functional status of the CL. For CL weight, with CL number included in the model as a covariate, a status × day interaction was detected ($P=0.05$) and reflected the decline in CL weight in cyclic ewes on day 16 that was not present in pregnant ewes. The reduction in CL weight was accompanied by declining plasma progesterone concentrations in cyclic ewes ($P<0.01$), which was in contrast to the gradually increasing plasma progesterone concentrations measured in pregnant ewes ($P<0.01$). In cyclic ewes, the negative linear relationship between plasma progesterone concentration and day suggests that hormone levels began to decline before there was evidence of reduced CL weight.

Endometrial oxytocin-stimulated formation of inositol phosphates was examined, in vitro, to provide an indirect estimate of the concentration of functional endometrial oxytocin receptors (Table 1).

![Figure 1](image1.png)

**Figure 1.** Total corpus luteum weight (g) in pregnant (solid bars) and cyclic (open bars) ewes (least squares means ± S.E.M.) on days 10, 12, 14 and 16 post-oestrus. Corpus luteum weight decreased by approximately 50% from day 14 to day 16 in cyclic ewes, but did not change during that period in pregnant ewes ($P=0.05$, status × day interaction).

![Figure 2](image2.png)

**Figure 2.** Plasma progesterone concentrations in pregnant (○) and cyclic (▼) ewes (least squares means ± S.E.M.) on days 10, 12, 14 and 16 post-oestrus. Regression analysis revealed that progesterone concentrations in the plasma of cyclic ewes decreased from day 10 to day 16 ($P<0.01$) but increased ($P<0.01$) in pregnant ewes.

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Oxytocin (100 nmol/l) was unable to stimulate the endometrial formation of inositol phosphates in vitro in pregnant ewes from day 10 to day 16, whereas, in cyclic ewes, endometrial sensitivity to oxytocin developed between days 14 and 16 (P<0.01).

Northern blot analyses were performed on endometrial poly(A)⁺ RNA isolated from one pregnant and one cyclic ewe on day 16, to determine the sizes of transcripts specifically hybridizing with the oestrogen receptor and progesterone receptor cDNA probes used for subsequent dot-blot analyses. The progesterone receptor cDNA hybridized with 3·1 and 1·6 kb transcripts and the oestrogen receptor cDNA hybridized with a 6·5 kb transcript (see Fig. 3). In agreement with dot-blot analysis, the relative amount of oestrogen receptor mRNA was lower in endometrium from pregnant than from cyclic ewes (Fig. 3).

Results from the densitometric analysis of radiographs for endometrial progesterone receptor mRNA are presented in Fig. 4. Analysis of variance in the amount of mRNA revealed distinct curvilinear (quadratic) relationships between the amount of progesterone receptor mRNA and the day of sampling between cyclic and pregnant ewes (pregnant: Y = -2·92 + 0·769X - 0·0345X²; cyclic: Y = -8·25 + 1·14X - 0·05X²; r² = 0·5, P < 0·01). The amount of progesterone receptor mRNA was greatest at days 10 and 12 of pregnancy and declined by day 16 (P < 0·05). In cyclic ewes, the amount of progesterone receptor mRNA was lowest at day 10 and increased to days 14 and 16 (P < 0·03). The amount of progesterone receptor mRNA was greater in pregnant ewes at day 10 (P < 0·01); however, by day 16 this relationship had reversed (P < 0·05).

Changes in endometrial progesterone receptor numbers are presented in Fig. 5. Analysis of variance revealed distinct curvilinear (quadratic) relationships between progesterone receptor numbers and day within each status (pregnant: Y = -4·4 + 1·02X - 0·042X²; cyclic: Y = 30·5 - 4·65X + 0·18X²; r² = 0·605, P < 0·01). In pregnant ewes, progesterone receptor numbers changed little from day 10 to day 16 (P > 0·10). In contrast, in cyclic ewes progesterone receptor numbers decreased by more than half from days 10 to 12 (P < 0·02), and then increased threefold by day 16 (P < 0·01). Endometrial progesterone receptor numbers were not different between pregnant and cyclic ewes on days 10 and 12, were greater in pregnant ewes on day 14, but were greater in cyclic ewes on day 16 (P < 0·05).

Changes in endometrial oestrogen receptor mRNA are presented in Fig. 6. Analysis of variance revealed distinct curvilinear (cubic) relationships between the amount of oestrogen receptor mRNA and day for cyclic and pregnant ewes (pregnant: Y = -15·5 + 36·88X - 2·82X² + 0·0704X³; cyclic: Y = -37·02 + 9·OX - 0·716X² + 0·0193X³; r² = 0·66, P < 0·01). In pregnant ewes, the amount of endometrial oestrogen receptor mRNA increased to a maximum between days 10 and 12 (P < 0·03), and decreased dramatically from day 12 to day 16 (P < 0·01). In cyclic ewes the amount of endometrial oestrogen receptor mRNA was lowest at day 10 and increased to day 16 (P < 0·01). The amount of oestrogen receptor mRNA was greater in pregnant ewes at days 10 (P = 0·05) and 12 (P < 0·05), not different at day 14 and lower at day 16 (P < 0·02), compared with cyclic ewes.

Changes in endometrial oestrogen receptor numbers are presented in Fig. 7. Analysis of variance revealed distinct curvilinear (quadratic) relationships between oestrogen receptor numbers and day for pregnant and cyclic ewes (pregnant: Y = 6·27 + 0·21X - 0·029X²; cyclic: Y = 118·07 - 19·39X + 0·814X²; r² = 0·75, P < 0·01). In pregnant ewes, endometrial oestrogen receptor numbers decreased from day 10 to day 16 (P < 0·03). In cyclic ewes, endometrial oestrogen receptor numbers were not different from day 10 to day 14, and were similar to those in pregnant ewes. However, oestrogen receptor numbers increased dramatically from day 14 to day...
FIGURE 3. Northern analysis of endometrial poly(A)+ RNA (25 μg) isolated from a cyclic (lanes 1 and 2) and a pregnant (lanes 3 and 4) ewe on day 16 post-oestrus, separated on a 1.5% agarose-formaldehyde gel and transferred to a nylon membrane. Gel-purified progesterone and oestrogen receptor cDNAs labelled with [32P]dCTP specifically hybridized with 1.6 and 3.1 kb transcripts (lanes 2 and 4) for the progesterone receptor cDNA and a 6.5 kb transcript (lanes 1 and 3) for the oestrogen receptor cDNA. The figure is a superposition of two radiographs from the same Northern blot which was first probed with the oestrogen receptor cDNA then boiled and reprobed with the progesterone receptor cDNA. Identical lanes are adjacent (i.e. 1 and 2, 3 and 4). The positions of rRNAs (28S and 18S) are indicated.

16 in cyclic ewes (P<0.01), being approximately ninefold greater than in pregnant ewes on day 16 (P<0.02).

Correlations between the amounts of endometrial oestrogen receptor mRNA and progesterone receptor mRNA (r=0.68), oestrogen receptor mRNA and oestrogen receptor protein (r=0.50) and oestrogen receptor and progesterone receptor protein (r=0.50) were high and positive (P<0.01). Amounts of endometrial progesterone receptor mRNA and protein, progesterone receptor mRNA and oestrogen receptor protein, and oestrogen receptor mRNA and progesterone receptor protein were not correlated. When data were adjusted for the effects of status, day and the interaction of status×day, the partial correlation between oestrogen receptor mRNA and progesterone receptor mRNA remained high and positive (partial r=0.57, P<0.01), whereas the correlations between oestrogen receptor mRNA and protein (r=0.24, P=0.31) and oestrogen receptor and progesterone receptor protein (r=−0.25, P=0.29) were no longer significant.

DISCUSSION

Results from the present study are the first to describe simultaneous measurements of mRNA and protein for oestrogen receptors and progesterone receptors in ovine endometrium during the period of maternal recognition of pregnancy and luteolysis. Measuring both the steady-state amounts of mRNA and levels of the steroid hormone receptors provides evidence for transcriptional, translational and post-translational control during luteolysis and early
pregnancy. For pregnancy to be maintained in ewes, conceptus secretory products must either extend the period of uterine responsiveness to progesterone or mimic the actions of progesterone on the uterine endometrium to inhibit luteolysis. The present study provides evidence for the former mechanism.

In cyclic ewes, the amount of endometrial progesterone receptor mRNA increased from day 10 to days 14–16, suggesting that the endometrium is in a transition from progesterone dominance to oestrogen dominance. It has been demonstrated in a number of species that, under the influence of oestrogen, both progesterone receptor mRNA (Savouret et al. 1991; Chauchereau et al. 1992) and progesterone receptor protein levels increase (Milgrom et al. 1972; Leavitt et al. 1974; Aronica & Katzenellenbogen, 1991). In the rat (Aronica & Katzenellenbogen, 1991), guinea-pig (Milgrom et al. 1972; Isotalo et al. 1981), hamster (Leavitt et al. 1974; Okulicz, 1986), primate (McClellon et al. 1990), rabbit (Quirk & Currie, 1984), cow (Meyer et al. 1988) and sheep (Miller et al. 1977b) uterus, oestrogen induces an increase in levels of the progesterone receptor. The highest levels of progesterone receptor coincide with maximum circulating oestrogen concentrations, and the lowest levels occur late in dioestrus or during implantation and early pregnancy for mated animals (Milgrom et al. 1972). During dioestrus, the effects of progesterone on the endometrium are characterized as 'antioestrogenic' (Hsueh et al. 1976; Read et al. 1988), but progesterone's 'antioestrogenic' actions occur only in uteri with elevated progesterone receptors (Walters & Clark, 1979). Doses of oestrogen that would result in luteolysis if given later in the cycle do not result in luteal regression during dioestrus (Hawk & Bolt, 1970; Warren et al. 1973; McCracken, 1980). This period of endometrial dominance by progesterone has been characterized by McCracken et al. (1984) as the 'progesterone block' to oxytocin receptor formation.

Down-regulation by progesterone of its own receptor provides a self-limiting luteal phase in a number of species (Leavitt et al. 1974; Miller et al. 1977b; Zelinski et al. 1980; Meyer et al. 1988; Clarke, 1990). A primary role for progesterone in regulating the length of the luteal phase has been demonstrated in experiments where progesterone was administered to sheep to stimulate an earlier rise in plasma progesterone than would be considered normal. By administering progesterone (40 mg/day) 8 or 32 h after the onset of oestrus, both the time to the first prostaglandin F2α peak (8 vs 12–3 days) and inter-oestrous intervals (11 vs 17 days) were decreased when compared with control ewes (Ottobre et al.
1980). In addition, ewes characterized by consistently short (13–14 days) oestrous cycles had an earlier rise in plasma progesterone concentrations than ewes exhibiting oestrous cycles of normal lengths (16–18 days; Nephew et al. 1991). Together, these results suggest that a period of approximately 10 days of elevated progesterone is necessary for down-regulation of the progesterone receptor and subsequent activation of the luteolytic mechanism.

For pregnancy to be established, the endometrium must remain responsive to progesterone (Milgrom et al. 1972; Warren et al. 1973; Koligian & Stormshak, 1977; McCracken et al. 1984; Cherny et al. 1991). Results from the present study demonstrate, for pregnant ewes, that although the amount of endometrial progesterone receptor mRNA decreased by approximately 50% from day 12 to day 16, progesterone receptor numbers changed little during this period. The fact that progesterone receptor mRNA levels declined during this period supports the hypothesis that the endometrium continues to be responsive to progesterone (Clarke, 1990), and the fact that both CL weight and plasma progesterone concentrations are maintained in pregnant, but not cyclic, ewes agrees with this contention. Progesterone receptor numbers changed little between days 10 and day 16 in spite of reduced levels of progesterone receptor mRNA. Perhaps secretions from the conceptus, or progesterone itself, directly or indirectly affect translational events, possibly increasing the number of active ribosomes, rate of peptide elongation or half-life of the receptor.

Results from this study demonstrate that the gestational endometrium is less dependent on oestrogen action than is found during the cycle, perhaps through a pregnancy-specific reduction in levels of oestrogen receptor protein and mRNA. Increases in endometrial oestrogen receptor protein and mRNA during the cycle were followed closely by increases in progesterone receptor protein and mRNA. However, pregnancy was marked by a 3- to 5-fold decrease in oestrogen receptor protein and mRNA, while only a 1.6- to 2-fold change occurred in progesterone receptor protein and mRNA. Thus, steady-state levels of progesterone receptor mRNA and the net synthesis of progesterone receptors do not appear to be as closely coupled to oestrogen action as they are during the cycle. These results are consistent with others suggesting that progesterone may either directly or indirectly maintain progesterone receptors during pregnancy (Maslar & Lazar, 1989; Ogle et al. 1990). Therefore, a pregnancy-specific reduction in oestrogen receptor protein and mRNA may help to explain how the endometrium remains responsive to progesterone, which it must if pregnancy is to be maintained, in spite of continuous exposure to elevated concentrations of progesterone.

In the cyclic ewe, progesterone blocks the ability of oestrogen to induce endometrial oxytocin receptor synthesis for about 10 days (McCracken, 1980; McCracken et al. 1984). After this, physiological doses of oestrogen result in increased oxytocin receptor synthesis, increased oxytocin-induced formation of inositol phosphates and a pulsatile pattern of prostaglandin F$_{2\alpha}$ production by the endometrium (McCracken et al. 1984; Leavitt et al. 1985; Bazer et al. 1991). Therefore, increases in oestrogen receptors in the endometrium may be necessary to up-regulate oxytocin receptors adequately, to elicit the luteolytic pulses of prostaglandin F$_{2\alpha}$ responsible for CL regression. This would not preclude oestrogen affecting post-receptor events in oxytocin-stimulated prostaglandin F$_{2\alpha}$ production, such as receptor coupling to a second messenger (Bouvier et al. 1991).

Oxytocin-stimulated endometrial inositol phosphate formation is correlated positively with the number and affinity of oxytocin receptors on the endometrium in cyclic ewes (Mirando et al. 1993). Formation of inositol phosphates by endometrium exposed to oxytocin was used as an indirect estimation of oxytocin receptors on the endometrium in the present study. In agreement with direct
measurements of endometrial oxytocin receptors (McCracken et al. 1984; Sheldrick & Flint, 1985), endometrium from pregnant ewes in this study was unresponsive to oxytocin from day 10 to day 16. In cyclic ewes, endometrial oxytocin-stimulated formation of inositol phosphates increased dramatically from day 14 to day 16, coincident with the decline in CL weight, suggesting that the luteolytic mechanism within the endometrium was functioning. One explanation for the apparent difference in endometrial sensitivity to oxytocin between cyclic and pregnant ewes is the greater number of endometrial oestrogen receptors in cyclic ewes, which may increase endometrial sensitivity to oxytocin during luteolysis. That oestrogen has a primary role in amplifying the mechanism responsible for luteolytic pulses of prostaglandin F₂₀ and timely CL regression is supported by the observation that destruction of developing follicles delays luteolysis in sheep (Hixon et al. 1975; Zhang et al. 1991).

Perhaps the most dramatic effect of pregnancy in the present study was on the oestrogen receptors. The presence of a conceptus suppressed both oestrogen receptor mRNA and protein in the endometrium. In cyclic sheep (Koligian & Stormshak, 1977; Miller et al. 1977b; Findlay et al. 1982; Cherny et al. 1991) and cattle (Kimball & Hansel, 1974; Senior, 1975; Zelinski et al. 1980; Meyer et al. 1988), oestrogen receptor levels are greatest during oestrus and decline under the influence of chronically elevated progesterone during dioestrus. For endometrial oestrogen receptors, the relative amounts of mRNA and protein were approximately five- and eightfold lower respectively in pregnant compared with cyclic ewes on day 16 post-oestrus. It is interesting that in cyclic ewes the amount of endometrial oestrogen receptor mRNA increased steadily from day 10 to day 16, but oestrogen receptor numbers were not different from day 10 to day 14, and only increased between days 14 and 16. One interpretation of these results is that during mid-dioestrus in the cyclic ewe progesterone inhibits oestrogen receptor accumulation by inhibiting both transcription and translation of the oestrogen receptor (Evans & Leavitt, 1980). During late dioestrus, as circulating progestins inhibit the responsiveness of the uterus to progesterone (by down-regulating the progesterone receptor), inhibition of oestrogen receptor transcription is lost first and oestrogen receptor mRNA accumulates (see Fig. 6) before the inhibition of translation of oestrogen receptor mRNA is lost (see Fig. 7). In this regard, day 15 post-oestrus appears to be a critical period for this transition.

Cherny et al. (1991) used immunocytochemistry to localize oestrogen receptors in the endometrium of cyclic and pregnant ewes. In agreement with results from this report, oestrogen receptors were suppressed in all regions of the endometrium at the end of the luteal phase (days 12–13). On day 15 of the cycle, oestrogen receptor staining was strong in all areas of the endometrium, whereas in pregnant ewes on day 15 oestrogen receptor staining was very weak or absent in all areas except for the deep caruncular stroma (Cherny et al. 1991).

The amounts of endometrial mRNA for the oestrogen and progesterone receptors showed a strong positive correlation. This correlation persisted even after the data were adjusted for the effects of status (pregnant vs cyclic), day and the status × day interaction. Endometrial oestrogen receptor mRNA and protein, as well as oestrogen receptor and progesterone receptor proteins, showed strong positive correlations which were consistent between statuses and across days; however, within status × day groups there was not a consistent relationship, and the limited observations within this grouping prevent strong conclusions from being made about the relationships. Progesterone receptor mRNA and progesterone receptor were not correlated, supporting our hypothesis that, in pregnancy, products of the conceptus intervene to stabilize progesterone receptor numbers (perhaps by regulating translational and/or post-translational events), thereby preserving uterine responsiveness to progesterone during the critical period when the luteolytic mechanism could be activated.

The steroid hormone receptor cDNAs used in this study specifically hybridized with a 6·5 kb transcript for the oestrogen receptor and 3·1 and 1·6 kb transcripts for the progesterone receptor. Both 6·6 and 6·2 kb transcripts for the oestrogen receptor were detected in human breast cancer cell lines (Read et al. 1989; Ree et al. 1991) and a 6·6 kb message was detected in porcine endometrium (R. D. Geisert, personal communication). The 7·5 or 7·8 kb oestrogen receptor transcripts found in chicken oviduct (Maxwell et al. 1987) were not detected. For progesterone, the 3·1 and 1·6 kb transcripts probably correspond to the 2·7 and 1·65 kb transcripts reported for porcine endometrium (Simmen et al. 1988). The importance of the 1·6 kb progesterone receptor transcript is equivocal because, presumably, it could not code for the entire receptor protein. Although the relative changes in the two transcripts were not determined, the low abundance of the 1·6 kb transcript relative to the 3·1 kb transcript (see Fig. 3) would preclude it from accounting for the relatively large (>50%) changes in amount of progesterone receptor mRNA measured across days in pregnant and cyclic ewes.

In cyclic ewes, endometrial oestrogen and progesterone receptor mRNA and protein and oxytocin-stimulated formation of inositol phosphates

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increased during luteolysis as plasma progesterone concentrations and CL weights declined. Pregnancy resulted in the stabilization of progesterone receptor numbers despite the declining steady-state amounts of progesterone receptor mRNA. There was also a decrease in the steady-state amounts of oestrogen receptor mRNA and protein and failure of the endometrium to respond to oxytocin in vitro. Results of this study provide support for the hypothesis that pregnancy alters the relationships between the steroid hormone receptors during the period of maternal recognition of pregnancy and, through this mechanism, may abrogate the increase in endometrial oestrogen and oxytocin receptors which is necessary for luteolysis in sheep.

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