Growth of the endometrium and cotyledons during pregnancy in the ewe: rates of protein secretion and synthesis and nuclear and cytosol steroid hormone receptor levels

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SUMMARY

The time-course of cell hypertrophy and changes in in-vitro rates of secretion and synthesis of protein in intercaruncular and caruncular endometrium and maternal and fetal cotyledonary placenta have been examined during days 0–112 of pregnancy in the ewe. The concentrations of high-affinity receptors for oestradiol and progesterone in nuclear and cytosol fractions from these tissues were also determined.

Protein secretion by intercaruncular endometrium increased 25-fold between days 0 and 84. On day 84 $10^{-5}$ M-colchicine blocked 75% of total secretion. Protein secretion did not increase in the other tissues. Protein synthesis and RNA : DNA ratio in intercaruncular endometrium increased steadily between days 0 and 112, whereas they did not change in caruncular endometrium between days 0 and 28 and declined in cotyledon between days 56 and 112. The levels of cytosol receptor for oestradiol and progesterone and of nuclear receptor for oestradiol in all tissues during days 56–112 were very low in relation to the corresponding levels in caruncular endometrium on day 0. The level of nuclear progesterone receptor in caruncular endometrium increased threefold between oestrus and day 28. The level of this receptor in cotyledon remained low on days 56–112, but in intercaruncular endometrium it increased to high values on days 84–112.

The results demonstrated a major surge in secretory activity by the intercaruncular endometrium at around mid-gestation, which was associated with a marked increase in nuclear progesterone receptor levels but only a low level of nuclear oestradiol receptor. The observations do not suggest any important role for oestradiol or progesterone in the growth of fetal and maternal cotyledon.

INTRODUCTION

The factors which regulate endometrial and cotyledonary placental growth and function during pregnancy in the sheep remain unclear. In a previous study changes in the in-vitro rates of protein synthesis and RNA : DNA and protein : DNA ratios in intercaruncular endometrium and fetal cotyledon during days 0–112 of pregnancy were compared with changes in the concentrations of high-affinity cytosol receptors for oestradiol and progesterone in whole uterus and maternal cotyledon. Rapid growth of these tissues between days 28 and 84 occurred in the presence of tissue levels of both cytosol steroid receptors that were extremely low in relation to the corresponding levels seen in the endometrium at oestrus (Miller & Stone, 1981). However, the interpretation of these data for cytosol receptors was made difficult by the absence of parallel data for nuclear oestradiol and progesterone receptor levels in these tissues. Nuclear receptor measurements
presumably give a better indication of the level of steroid stimulation occurring in these tissues (Jensen, 1979). Further, not all of the principal endometrial and cotyledary tissues were examined.

The aims of this more detailed study of endometrial and placental growth and function were (1) to examine in the intercaruncular and caruncular endometrium and in maternal and fetal cotyledon changes in the in-vitro rates of protein secretion, in the presence and absence of colchicine, an inhibitor of secretory processes (Wolff & Williams, 1973); as well as changes in in-vitro protein synthesis and tissue RNA : DNA and protein : DNA ratios and (2) to relate, where possible, these events to corresponding changes in both nuclear and cytosol levels of oestradiol and progesterone receptors.

**MATERIALS AND METHODS**

**Animals**

Twenty-five mature Merino ewes on pasture received Repromap intravaginal sponges (60 mg medroxyprogesterone acetate; Upjohn Pty Ltd, Rydalmere, New South Wales, Australia). The sponges were removed 14 days after insertion and ewes were joined with rams bearing marking crayons. The day on which mating first occurred was designated day 0. Mated ewes were allotted to be killed on days 0, 28, 56, 84 and 112 of pregnancy at random. Each ewe was killed by prompt sectioning of the principal cervical blood vessels and cervical spinal cord at about 08.30 h; the genital tract was promptly removed and packed in crushed ice.

**In-vitro protein synthesis and secretion, and RNA : DNA and protein : DNA ratios**

Small portions (100–200 mg) of intercaruncular or intercotyledary endometrium were collected with fine scissors. On days 28–112 the loosely adherent fetal trophoblast or chorioallantois was peeled away from the intercaruncular endometrium before tissue collection. At days 0 and 28 slices of caruncular endometrium were obtained using a Stadie–Riggs microtome (Miller, Murphy & Stone, 1977). At days 56–112 the spongy fetal cotyledon was bluntly dissected from the embracing and more fibrous maternal cotyledon (endometrial capsule, burl). Small portions of tissue (100–200 mg) were collected from the opposing surfaces of the fetal and maternal cotyledons exposed by this blunt dissection. Each of these cotyledonic tissues contains cells of both maternal and fetal origin (for discussions of the gross and fine structure of the ovine placenta, see Amoroso, 1952; Davies & Wimsatt, 1966; Steven, 1975). All tissue preparation was carried out at 0–3°C.

Twelve pieces of each tissue were collected from each ewe. These were allotted at random to four 25 ml Erlenmeyer flasks containing three tissue pieces each. The tissues in each flask were incubated at 37°C in 2 ml Eagle’s (Basal) medium under an atmosphere of 95% O₂ : 5% CO₂. The medium in two only of each set of four flasks was supplemented with 10⁻² m-colchicine (Sigma, St Louis, Missouri, U.S.A.). Tissues were preincubated for 2 h, then the medium was supplemented with 3 µCi l-[4,5-³H]leucine (1 Ci/mmol, Amersham International Ltd, Bucks), and incubations were continued for a further 4 h. At the end of the incubations tissues were promptly removed from the incubation medium, washed in ice-cold 0·9% NaCl (w/v) solution and stored at −18°C. The incubation medium was chilled to 5°C and centrifuged at 105 000g for 60 min. The supernatant fraction obtained was supplemented with 50 µl sheep serum and proteins were precipitated by the addition of 1·0 ml 1·0 m-HClO₄ and collected by centrifugation at 1000g for 15 min. The pellet obtained was washed three times with 5 ml 0·2 m-HClO₄, then solubilized by heating and stirring at 50°C for 15 min in 0·75 ml 0·25 m-NaOH, and samples (200 µl) were taken for liquid scintillation counting. The methods used to determine RNA : DNA and protein : DNA ratios and the incorporation of tritium into tissue and secreted protein have been described (Miller, 1979).
Mean rates of incorporation of tritium into tissue and secreted proteins were expressed as d.p.m. $^3$H/$\mu$g DNA. Rate of protein synthesis was determined by summing the rates of tritium incorporation into tissue and secreted proteins. Tritium incorporation into secreted protein in the absence of $10^{-5}$ m-colchicine gave the total secretion rate, and the difference between tritium incorporation into secreted protein in the presence and absence of colchicine gave the rate of colchicine-sensitive secretion. Preliminary experiments examined the kinetics of tritium incorporation into the tissue and secreted protein fractions and the effects of colchicine on these parameters. Increasing concentrations of colchicine up to $10^{-4}$mol/l decreased tritium incorporation in secreted proteins, and incorporation of tritium into both protein fractions in the presence or absence of colchicine was linear for at least 4 h.

**Chemicals and buffers for receptor assay**

[6,7-$^3$H]Oestradiol (sp.act. 54 Ci/mmol) and [1,2-$^3$H]progesterone (sp.act. 43 Ci/mmol) were obtained from Amersham International Ltd, while [17$\alpha$-methyl-$^3$H]promegesterone (R5020) (sp.act. 87 Ci/mmol) and unlabelled R5020 were obtained from New England Nuclear (Boston, Massachusetts, U.S.A.). All other chemicals were analytical grade. Aqueous samples were counted in toluene-Terix X-10 (2:1, v/v) containing 2.8 g diphenyloxazole (PPO)/l and 70 mg 1,4-bis-2-(5 phenyloxazolyl)-benzene (POPOP)/l, while non-aqueous samples were counted in toluene containing 3 g PPO and 100 mg POPOP/l. Buffer 1 contained 50 mM-Tris–HCl, 1 mM-EDTA, 12 mM-monothioglycerol and 30% glycerol (v/v), pH 7.5, and was used in the initial homogenization for nuclear receptors. Buffer 2 contained 10 mM-Tris–HCl, 1 mM-EDTA, 12 mM-monothioglycerol, pH 7.5, and was used to dissolve steroids for nuclear receptor assay, and for nuclear charcoal/dextran solutions. Buffer 3 was buffer 1 plus 0.5 M-KCl and was used for the extraction of nuclear receptor, and buffer 4 was buffer 2 containing 10% glycerol (v/v) and was used for washing the nuclear pellet before extraction. All these buffers were freshly prepared the day before use. For assays of the nuclear oestriadiol, but not nuclear progesterone receptor, they were supplemented with 10 mM-Na$$_2$$MoO$_4$ (Stone, McCaffery & Miller, 1982). Buffer 5 contained 0.25 M-sucrose, 10 mM-Tris–HCl, 1.5 mM-EDTA, 5 mM-NaF, 10 mM-Na$_2$MoO$_4$, 10% glycerol (v/v), pH 7.4, and was used for tissue homogenization in cytosol receptor assays. Buffer 6 was buffer 5 without sucrose and was used for cytosol charcoal/dextran solutions and to dissolve steroids for cytosol receptor assay. Dextran/charcoal solutions contained 5 g charcoal (Norit A; Mathesen, Coleman and Bell, Norwood, Ohio, U.S.A.) and 0.5 g dextran (T/70; Pharmacia, Uppsala, Sweden)/l for nuclear assays and 5.5 g charcoal and 0.55 g dextran/l for cytosol assays.

**Cytosol receptor assay**

Portions of caruncular endometrium (days 0–28) and intercaruncular endometrium and maternal and fetal cotyledon (days 56–112) were collected as described above and minced finely with scissors. Duplicate samples (0.5 g) were homogenized in 12 vol. buffer 5 using an Ultra-Turrax homogenizer (Janke and Kunkel, Staufen, West Germany) at 180 $\nu$ using 3 $\times$ 10 s bursts with 30 s between bursts. The homogenate was centrifuged for 10 min at 20000 $g$ and the pellet saved for DNA estimation. The final cytosol was prepared by centrifugation of the supernatant fraction for 60 min at 105000 $g$. Endogenous free or weakly bound steroid was removed with charcoal/dextran (10:1) for 10 min, removing the charcoal by centrifugation. The level of oestradiol and progesterone cytosol receptors was measured as described previously (Miller et al. 1977; Stone, Wild & Miller, 1979). In brief, for oestradiol receptor, samples of cytosol were incubated with [1$^H$]oestradiol (final concentration 10 nmol/l) in the presence or absence of a 100-fold excess of unlabelled diethylstilboestrol. Exchange with any endogenous bound steroid was tested at 30 $^\circ$C for 20 min. Free steroid was removed with charcoal as above and the receptor was measured.
after agar gel electrophoresis at low temperature (Wagner, 1972). For the progesterone receptor, samples were incubated with cortisol (final concentration 1 µmol/l) for 30 min at 0°C and were then incubated for 18 h at 0°C with [3H]R5020 (final concentration 10 nmol/l) with and without a 100-fold excess of unlabelled R5020. Incubates were then treated with charcoal and analysed by agar gel electrophoresis as for the oestradiol receptor.

Nuclear receptor assay

For both receptors the in-vitro exchange procedures of Chen & Leavitt (1979) and Evans, Chen, Hendry & Leavitt (1980) were essentially followed, as modified by Stone et al. (1982). Tissue was prepared as for the cytosol receptors and duplicate 0.5 g samples were homogenized in 10 vol. buffer 1. The homogenate was centrifuged at 800g for 15 min and the ‘nuclear pellet’ fraction washed three times with 10 vol. buffer 4, resuspending with a Teflon pestle. A sample of the final suspension was taken for DNA estimation. Nuclear receptor was extracted at 0°C from the final pellet using 7 vol. buffer 3 and resuspending every 5 min for 1 h. The extract was collected after centrifugation at 20000g for 40 min. Nuclear receptor was measured in triplicate 300 µl samples of the extract which were incubated with [3H]steroid (final concentration 10 nmol/l) with or without a 100-fold excess of the appropriate unlabelled compound (diethylstilboestrol or progesterone) to give a total volume of 500 µl. For oestradiol receptor, exchange was effected at 30°C for 60 min and for progesterone receptor at 0°C for 18 h. Free steroid was removed by a 1-min treatment with 500 µl charcoal solution.

Results for all receptors are expressed as pmol steroid bound per mg DNA. The diphenylamine method of Burton (1956) using calf thymus DNA as the standard was used to measure DNA. Because of the likely high level of endogenous steroid in these tissues, particularly of progesterone in fetal cotyledon, it was necessary to ensure that the proportion of this steroid in the subcellular fractions for receptor assay would not interfere with the assay. Tissues from an animal at day 112 of pregnancy were homogenized in buffer containing a trace of labelled oestradiol or progesterone and the proportion of the added radioactivity in the respective fractions measured. With an assumed endogenous concentration of 1 µg steroid/g of tissue, the level found in the fractions for assay was only a minor proportion of that added as label in the receptor assays.

Statistical analysis

Data were analysed by Duncan’s multiple range test (Steel & Torrie, 1960).

RESULTS

Data were obtained from four ewes on day 0, and from four pregnant ewes on each of days 28, 56, 84 and 112. Seven of the ewes killed on days 28–112 had twin fetuses; the remainder had single fetuses.

At oestrus the rate of secretion of protein by intercaruncular endometrium was approximately double that in caruncular endometrium, and in each tissue 10⁻⁵M-colchicine reduced secretion by about 50%. Between day 0 and peak secretion on day 84 the total secretion rate in intercaruncular endometrium increased 25-fold and colchicine-inhibited secretion increased 37-fold (Fig. 1a, b). The increases between days 0 and 28, 28 and 56, and 56 and 84 were all highly significant (P<0.01). No comparable gestational responses occurred in caruncular endometrium or cotyledonary tissues. Rather, total secretion rates in maternal cotyledon on days 56–112 were only about one-half of those in caruncular endometrium on days 0–28, and the proportion of protein secretion inhibited by colchicine decreased to 22–30%. Secretion rates in fetal cotyledon on days 56–112 remained similar to those in caruncular endometrium on days 0–28.
The in-vitro rate of protein synthesis in all tissues was not altered by the inclusion of $10^{-5}$ M-colchicine in the incubation medium (Fig. 1c, d). At oestrus the rate in intercaruncular endometrium was approximately twice the rate in caruncular endometrium, and at this time rates of protein synthesis were about forty-five times higher than the corresponding rates of protein secretion. Protein synthesis increased steadily in intercaruncular endometrium during pregnancy to rates at days 84–112 which were two- to threefold greater than at oestrus ($P<0.01$). In contrast, the rate in caruncular endometrium did not change between days 0 and 28, and between days 56 and 112 protein synthesis declined in maternal ($P<0.05$) and fetal ($P<0.01$) cotyledon.

At oestrus RNA : DNA and protein : DNA ratios were slightly higher in intercaruncular than in caruncular endometrium (Table 1). The ratios in intercaruncular endometrium increased slowly to day 28, then more rapidly between day 28 and days 84–112 ($P<0.01$).
Table 1. Effect of the stage of pregnancy on RNA:DNA and protein:DNA ratios in endometrial and cotyledonary tissues of the ewe. Values are means ± S.E.M. of four animals.

<table>
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<tr>
<th>Day of pregnancy</th>
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<tr>
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<td>Caruncular</td>
<td>Maternal</td>
<td>Fetal</td>
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<td>RNA : DNA</td>
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<tr>
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<tr>
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<td>0-429 ± 0-021</td>
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<tr>
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<td>0-999 ± 0-158</td>
<td>—</td>
<td>1-053 ± 0-034</td>
<td>1-136 ± 0-060</td>
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<tr>
<td>84</td>
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<tr>
<td>112</td>
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<td>0-615 ± 0-040</td>
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<td>—</td>
<td>12-90 ± 0-55</td>
<td>10-32 ± 0-63</td>
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</table>

Fig. 2. Effect of the stage of pregnancy in ewes on (a, b) oestradiol and (c, d) progesterone receptor concentration in (a, c) cytosol and (b, d) nuclear fractions of intercaruncular endometrium (●), days 56, 84, 112, caruncular endometrium (○), days 0, 28, maternal cotyledon (○), days 56, 84, 112 and fetal cotyledon (●), days 56, 84, 112. Results are expressed as pmol steroid bound mg tissue DNA. For further explanation see Materials and Methods. Vertical bars indicate one S.E.M.; standard errors which are not shown fall within the points.
Those for caruncular endometrium did not change. In maternal and fetal cotyledon the RNA : DNA ratio declined during days 56–112 ($P<0.01$), whereas the protein : DNA ratio remained fairly constant.

Steroid receptor levels were not determined in intercaruncular endometrium on days 0 and 28, due to lack of adequate tissue. The level of oestradiol receptor in both cytosol and nuclear fractions of caruncular endometrium was high at oestrus and declined markedly ($P<0.01$) to day 28 (Fig. 2a, b). In both maternal cotyledon and intercaruncular endometrium the level of cytosol oestradiol receptor declined between days 56 and 112 ($P<0.05$) and at all times was less than the level in caruncular endometrium on day 28. Very little cytosol oestradiol receptor was detected in fetal cotyledon. In the case of the nuclear oestradiol receptor, the levels on days 56–112 in all tissues were always low in relation to those in caruncular endometrium on day 28.

The level of cytosol progesterone receptor in caruncular endometrium decreased markedly ($P<0.01$) between oestrus and day 28, and thereafter the levels in all tissues were low in relation to those in caruncular endometrium on day 28 (Fig. 2c). The levels in fetal cotyledon were especially low. The pattern of change in nuclear progesterone receptor level during pregnancy was quite different (Fig. 2d). In caruncular endometrium the level increased about threefold between oestrus and day 28 ($P<0.01$). On day 56 the levels in all tissues were similar to the level in caruncular endometrium at oestrus, but the changes occurring after day 56 were dissimilar. In maternal and fetal cotyledon the changes in nuclear progesterone receptor level were not significant, while in intercaruncular endometrium the level increased about threefold ($P<0.01$) to high values on days 84–112, presumably reflecting increased translocation of receptor to the nucleus at these times.

**DISCUSSION**

Many inhibitory effects of colchicine on secretory processes have been described and are probably caused by the binding of colchicine to the dimeric unit of microtubules causing their disaggregation. In tissues that secrete protein colchicine has little effect on protein and RNA synthesis in concentrations which readily block microtubule function (Wolff & Williams, 1973). Colchicine uptake into tissues occurs slowly, and once the colchicine effect on secretion is established it is not easily reversed. The selection of a 2-h preincubation period with $10^{-5}$ M-colchicine was based on results for colchicine effects on thyroidal $^{131}$I release (Wolff & Williams, 1973). Colchicine blocked 80% of $^{131}$I release under these conditions, but longer preincubation periods blocked higher proportions of $^{131}$I release. Therefore the results for colchicine sensitivity presumably give a conservative estimate of the amount of true secretion.

The present results for changes during pregnancy in RNA : DNA and protein : DNA ratios and in-vitro protein synthesis in intercaruncular and caruncular endometrium and fetal cotyledon agree well with our earlier findings (Miller & Stone, 1981). Changes in maternal cotyledon during days 56–112 were not previously examined. The changes occurring in maternal and fetal cotyledon during this interval were similar. Rates of in-vitro protein synthesis and RNA : DNA ratios in both cotyledonary tissues declined substantially after day 56, whereas protein : DNA ratios appeared to decline only after day 84. These data suggest that the decline in rate of intracellular protein synthesis in cotyledons precedes by several weeks the progressive decrease from about day 90 in the volume of connective tissues which forms the core of the placental villi (Barcroft & Barron, 1946). In comparison with fetal cotyledon, the protein : DNA ratio was always higher in maternal cotyledon, the RNA : DNA ratio was about the same and the rate of protein synthesis was lower.

Total protein secretion by the intercotyledonary endometrium declined between days 0 and 10 (B. G. Miller, unpublished data) then increased, most rapidly between days 56 and
84. Secretion appeared maximal at around day 84. Moffatt, Bazer, Caton & Roberts (1980) have examined endometrial protein secretion in the ewe. Although the increase they observed during pregnancy was proportionately smaller, most of the increase occurred between days 60 and 90, in close agreement with the present results. Endometrial protein secretion in the pregnant ewe is evidently regulated by progesterone and can be approximately simulated in non-pregnant ewes by auto-transplantation of an ovary containing a functional corpus luteum (Harrison, Heap, Horton & Poyser, 1972) or by giving progesterone for 120 days (Moffatt et al. 1980). As regards this probable role of progesterone, it is interesting to note that the period of rapidly increasing protein secretion coincides with that of rapidly increasing placental progesterone synthesis (Ricketts & Flint, 1980) and rising peripheral plasma progesterone levels (Bassett, Oxborrow, Smith & Thorburn, 1969; Tsang, 1978). The function of these secreted proteins in the pregnant ewe is unknown. After implantation the endometrial glands increase in length and complexity. Those portions of the chorioallantois facing the mouths of glands become regionally specialized to form absorptive 'areolae'. At around days 80–100 a thick accumulation of finely granular material is observed within the uterine lumen, which is presumably a secretory product of the uterine glands (Amoroso, 1952; Davies & Wimsatt, 1966; Perry, 1981).

The low levels of in-vitro protein secretion occurring in cotyledonary tissues on days 56–112 suggest that the secretion of placental lactogen and perhaps other protein hormones by fetal elements of the cotyledon (Boshier & Holloway, 1977; Wooding, 1981) is quantitatively trivial in relation to protein secretion by the intercaruncular endometrium.

The results for cytosol receptors for oestradiol and progesterone show a similar pattern of change in all the tissues studied, i.e. a decrease from high oestrous levels to low levels similar to those previously described for whole uterus and caruncles/cotyledons (Miller & Stone, 1981). The active replenishment of cytosol progesterone receptor in rabbit uterus under conditions of chronic progesterone treatment (Isotalo, Isomaa & Jänne, 1981) and which would presumably be akin to the progesterone dominance of pregnancy, was not observed. Thus, in the pregnant ewe, the cytosol level of both receptors remains depressed under conditions of a high plasma (Bassett et al. 1969) and a high uterine (Rawlings & Ward, 1976) progesterone concentration. The nuclear oestradiol receptor level remained low after oestrus in all endometrial and cotyledonary tissues. Endometrial sulphatase activity in the ewe is low during pregnancy (Dwyer & Robertson, 1980) and unconjugated oestrogens are present in maternal plasma and endometrium in only very low concentrations during the first two-thirds of pregnancy (Robertson & Smeaton, 1973; Tsang, 1978).

Thus rapid growth and changes in function of the endometrium and placenta occur while the levels of oestradiol receptor, both cytosol and nuclear, and of cytosol progesterone receptor remain low. These results do not suggest any important role for oestradiol and/or its receptor in these tissues during days 28–112 of gestation. The changes in nuclear progesterone receptor content did differ between tissues and are of considerable interest. In caruncular endometrium there was a rise between days 0 and 28, possibly associated with the rise in plasma progesterone level (Bassett et al. 1969), but not associated with any change in tissue RNA : DNA or protein : DNA ratio or in protein synthesis or secretion. By day 56, when there was a marked hypertrophy in maternal cotyledon in relation to the caruncles on day 28, the nuclear progesterone receptor level had fallen and it subsequently remained low. On the other hand, the rapid rise in protein secretion rate in intercaruncular endometrium from day 56 onwards is accompanied by a marked rise in nuclear progesterone receptor level. This finding seems contrary to the observation that increasing uterine secretion of uteroglobin in the rabbit is associated with declining levels of nuclear progesterone receptor (Isomaa, 1981; Young, Smith & Bullock, 1981). The increasing nuclear progesterone receptor levels are evidently not explained by increasing oestrogenic
stimulation, since nuclear and cytosol oestradiol receptor levels remain low. It is tempting to suggest that rising nuclear levels of progesterone and/or its receptor control the secretion rate in intercaruncular endometrium.

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