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Hormone Receptor Levels and Metabolic Activity in the Uterus of the Ewe: Regulation by Oestradiol and Progesterone

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Abstract

Oestradiol and progesterone were administered alone or together in various combinations to different groups of ovariectomized ewes and several aspects of uterine and endometrial metabolism were determined. Levels of cytosol receptors for oestradiol and progesterone and metabolism of these steroids in whole uterus were measured, as well as mean cell content of RNA, rate of protein synthesis, tissue DNA concentration and alkaline phosphatase activity in the endometrium, uterine weight, and the amount of protein in uterine flushing.

Oestradiol increased the level of both receptors, the RNA : DNA ratio and the rate of protein synthesis, it decreased tissue DNA concentration but it had no effect on alkaline phosphatase activity. Progesterone increased the level of the oestradiol receptor, the RNA : DNA ratio, the rate of protein synthesis and alkaline phosphatase activity, it decreased tissue DNA concentration but it did not change the level of the progesterone receptor. There were few interactions between oestradiol and progesterone. However, in ewes which received oestradiol the administration of progesterone for 9 days caused small decreases in the level of the oestradiol receptor and larger decreases in the level of the progesterone receptor. None of the hormone treatments had significant effects on oestradiol or progesterone metabolism or on the amount of protein in uterine flushings. The results are discussed in relation to the possible roles of steroid receptors in the uterus, and to changes occurring in the uterus of the intact ewe during the oestrous cycle.

Introduction

During the luteal phase of the oestrous cycle the uterus of the ewe is exposed to high levels of circulating progesterone for about 9 days (Moore *et al.* 1969; Thorburn *et al.* 1969; Cunningham *et al.* 1975). The uterus is also exposed to high plasma levels of oestradiol for periods of 2–3 days. The largest surge of ovarian oestrogen secretion occurs during pro-oestrus and early oestrus when plasma progesterone levels are minimal, but two or three similar but smaller surges occur at intervals during the remainder of the oestrous cycle (Cox *et al.* 1971; Mattner and Braden 1972; Cox *et al.* 1973; Baird *et al.* 1976).

In a previous study (Miller *et al.* 1977*a*) we described the changes in steroid receptor levels and RNA and protein metabolism that occur in the uterus during the normal oestrous cycle. A similar pattern of change was observed in each uterine activity, with levels of cytosol receptors and rates of endometrial metabolism being maximal at or shortly after oestrus and minimal during the last 4 days of the luteal phase of the cycle. In other experiments (Murphy *et al.* 1977; Miller *et al.* 1977*b*) we have determined some effects on the uterus and on embryo development of complex dose regimes of oestradiol and progesterone which were intended to simulate endogenous ovarian secretion during the oestrous cycle and early pregnancy. However, it was

not possible to determine from these data to what extent the uterine changes which occur during the oestrous cycle were caused by the changing plasma levels of oestrogens or of progesterone.

In the present study employing ovariectomized ewes we have examined the effects on the uterus of nine simple dose schedules of oestradiol and/or progesterone. The treatments were designed to define the role of each hormone and to reveal any interactions or priming effects within the uterus which occur when the two hormones are administered simultaneously or sequentially. Results for hormone effects on cytosol concentration of oestradiol and progesterone receptors, rate of protein synthesis, mean cell content of RNA, uterine weight, endometrial cell density and alkaline phosphatase activity, the amount of protein in uterine flushings, and uterine metabolism of [^{3}H]oestradiol and [^{3}H]progesterone are described.

Materials and Methods

Animals and Schedule of Hormone Injections

Replicate experiments were performed in May and November 1976. For each replicate 30 mature Merino ewes were ovariectomized 4 weeks before day 1 of the experiment and received a single priming injection of 40 μg oestradiol 2 weeks later. Ewes were allotted at random to 10 groups each of three animals. Control ewes received no injections (group 1). Three basic treatments were examined: oestradiol given for 3 days (40 μg/day), and progesterone given for 3 or 9 days (12 mg/day) (groups 2–4). In the remaining groups (5–10) various combinations of these three treatments were given, and some ewes (groups 9 and 10) received injections on each of days 1–12. The schedule of doses and days of injection are shown in Table 1. Hormones were administered intramuscularly in 1·0 ml peanut oil, and all ewes were killed on day 14. The injections of hormones into different ewes were staggered, so that 10 ewes were killed on each of three consecutive days. Ewes were killed by exsanguination, and the genital tracts were promptly dissected and packed in crushed ice.

Table 1. Schedule of oestradiol and progesterone injections given to ovariectomized ewes

All injections were given between 0800 and 0900 h and all ewes were killed between 0900 and 1030 h on day 14. E_2, 40 μg oestradiol; P, 12 mg progesterone

Group of ewes	Treatment given on day: 1	2	3	4	5	6	7	8	9	10	11	12
1	—	—	—	—	—	—	—	—	—	—	—	—
2	—	—	—	—	—	—	—	—	—	E_2	E_2	E_2
3	—	—	—	—	—	—	—	—	—	P	P	P
4	—	—	—	P	P	P	P	P	P	P	P	P
5	—	—	—	—	—	—	—	—	—	E_2+P	E_2+P	E_2+P
6	—	—	—	—	—	—	P	P	P	E_2	E_2	E_2
7	—	—	—	—	—	—	E_2	E_2	E_2	P	P	P
8	—	—	—	P	P	P	P	P	P	E_2+P	E_2+P	E_2+P
9	E_2	E_2	E_2	P	P	P	P	P	P	P	P	P
10	P	P	P	P	P	P	P	P	P	E_2	E_2	E_2

Estimation of Total Protein in Uterine Flushings

Each chilled uterus was flushed with 20·0 ml ice-cold 0·15 M NaCl. The solution was injected into the lumen near the utero–tubal junction of one uterine horn and collected through a polythene cannula inserted into the lumen at the utero–tubal junction of the opposite horn. The flushings were centrifuged at 12000 g for 30 min at 5°C. After decanting, the volume of the supernatants was adjusted to 20·0 ml with 0·15 M NaCl and aliquots were taken to determine protein (Hartree 1972). Each uterus was then separated from adjacent tissues and from the cervix at the level of the internal os, and weighed.

Rates of Synthesis of Protein, and Tissue RNA : DNA Ratios

Slices of endometrium were prepared from dissected caruncles, and *in vitro* rates of synthesis of protein and RNA : DNA ratios were determined as previously described (Miller 1976), except for the following two modifications. To determine rates of protein synthesis each incubation was carried out in the presence of 1·0 μCi L-[4,5-^{3}H] leucine (1 Ci/mmol, Radiochemical Centre, Amersham, U.K.) instead of L-[methyl-^{3}H]methionine; and for each ewe RNA : DNA ratios and rates of synthesis of protein were determined in the same tissue samples, so that the results for protein synthesis could be expressed per microgram of tissue DNA. Duplicate determinations were carried out for each ewe and three endometrial slices were used in each duplicate. Results are expressed as mean ^{3}H dpm (protein) per microgram of DNA and mean tissue RNA : DNA ratios.

Endometrial Tissue DNA Concentration

Portions of uterus to be used for estimating cell density and alkaline phosphatase activity were stored at −28°C. After thawing, two portions of endometrium, each comprising two caruncles and the adjacent intercaruncular endometrium, were dissected from each uterus. Each portion was gently blotted, weighed, then homogenized in 6·0 ml ice-cold distilled water. The homogenate was acidified with 2·0 ml 1·0 M $HClO_4$ and centrifuged at 1000 *g* for 20 min at 5°C. The $HClO_4$-insoluble pellet was washed once with 5·0 ml 0·2 M $HClO_4$. DNA was extracted from the $HClO_4$-insoluble pellet by hydrolysis at 90°C for 20 min in 2·0 ml 0·5 M $HClO_4$, and was recovered in the supernatant obtained by chilling and centrifuging the sample at 1000 *g* for 20 min. DNA was determined by the method of Burton (1956) using calf thymus DNA as a standard. Results for these estimates of endometrial cell density are expressed as mean micrograms of DNA per milligram of endometrium.

Alkaline Phosphatase Activity in the Endometrium

Studies were confined to intercaruncular endometrium, since the enzyme activity in this tissue is 2–3 times greater than that in caruncles (Murdoch and White 1968*a*, 1968*b*). Preliminary studies showed that storage of uteri at −28°C for several weeks did not diminish endometrial alkaline phosphatase activity. Two portions of intercaruncular endometrium were collected from each uterus with fine scissors. Each portion was homogenized in 6·0 ml distilled water and centrifuged at 1000 g. Aliquots of the supernatant were taken to determine protein (Hartree 1972) and enzyme activity. Incubations were for 30 min at 37°C in 1·0 ml of buffer (50 mM glycine–NaOH, pH 10·5, containing 0·5 mM $MgCl_2$ and 6 mM p-nitrophenyl phosphate, Na_2 salt). The reaction was terminated by the addition of 2·0 ml 0·1 M NaOH. p-Nitrophenyl (Calbiochem, California, U.S.A.) was used as a standard. Results are expressed as mean micromoles of p-nitrophenol liberated per milligram of protein.

[^{3}H]Oestradiol and [^{3}H]Progesterone Metabolism and Assay of Cytosol Receptors for Oestradiol and Progesterone

Steroid metabolism by whole uterus minces in the first replicate was measured as previously described (Miller *et al.* 1977*a*). The level of total cytosol oestradiol and progesterone receptor was also measured as previously described (Miller *et al.* 1977*a*). Results for fresh whole uterus from the November replicate only are presented. Tissues from the initial replicate showed an as yet unexplained progressive decline in receptor content during their storage at −30°C.

Statistical Analysis of the Data

The significance of differences between treatments was examined by analysis of variance and Duncan's multiple range test (Steel and Torrie 1960). There were significant differences between replicates (May *v.* November) in some of the activities measured.

Results

Effect of Oestradiol (Group 2 v. *Group 1)*

Giving oestradiol on days 10–12 almost doubled the weight of the uterus and the rate of protein synthesis and mean cell content of RNA in the endometrium on day 14 ($P < 0·01$). Cytosol concentration of oestradiol and progesterone receptor proteins was increased about three- and four-fold respectively ($P < 0·01$). Endometrial cell

Table 2. Effects of progesterone and oestradiol on the uterus and endometrium of the ewe

Means with the same superscript are not significantly different at the 0·05% level. W.U., whole uterus; C.E., caruncular endometrium; I.C.E., intercaruncular endometrium; T.E., total endometrium

Group	Uterine wet weight[A] (g)	RNA : DNA[A] (μg/μg) C.E.	Protein synthesis[A] (^{3}H dpm/μg DNA) C.E.	Alkaline phosphatase[A] (μmol/mg protein) I.C.E.	DNA : wet weight[A] (μg/mg) T.E.	Oestradiol cytosol receptor[B] (pmol steroid bound/mg DNA) W.U.	Progesterone cytosol receptor[B] (pmol steroid bound/mg DNA) W.U.
1	$20{\cdot}0^{a}$	$0{\cdot}343^{a}$	$13{\cdot}5^{a}$	$3{\cdot}63^{a}$	$15{\cdot}86^{e}$	$4{\cdot}52^{a}$	$2{\cdot}87^{a}$
2	$36{\cdot}2^{c,d}$	$0{\cdot}614^{f,g}$	$26{\cdot}9^{b,c,d}$	$4{\cdot}30^{a}$	$9{\cdot}92^{b,c}$	$11{\cdot}80^{d,e}$	$12{\cdot}22^{e}$
3	$24{\cdot}4^{a,b}$	$0{\cdot}470^{b,c,d}$	$25{\cdot}9^{b,c}$	$13{\cdot}59^{d}$	$12{\cdot}19^{d}$	$8{\cdot}45^{b,c}$	$4{\cdot}71^{a,b,c}$
4	$34{\cdot}1^{c,d}$	$0{\cdot}450^{b,c}$	$22{\cdot}0^{b}$	$12{\cdot}18^{c,d}$	$11{\cdot}23^{c,d}$	$8{\cdot}20^{b,c}$	$3{\cdot}78^{a,b}$
5	$36{\cdot}2^{c,d}$	$0{\cdot}609^{f,g}$	$34{\cdot}4^{d}$	$8{\cdot}63^{b,c}$	$8{\cdot}34^{a,b}$	$12{\cdot}62^{d,e}$	$9{\cdot}05^{d}$
6	$42{\cdot}4^{d,e}$	$0{\cdot}539^{d,e,f}$	$26{\cdot}8^{b,c,d}$	$4{\cdot}89^{a,b}$	$8{\cdot}26^{a,b}$	$11{\cdot}16^{d,e}$	$11{\cdot}95^{e}$
7	$36{\cdot}0^{c,d}$	$0{\cdot}526^{c,d,e}$	$27{\cdot}8^{b,c,d}$	$11{\cdot}54^{c,d}$	$8{\cdot}99^{a,b}$	$10{\cdot}19^{c,d}$	$5{\cdot}65^{b,c}$
8	$41{\cdot}6^{d,e}$	$0{\cdot}666^{g}$	$32{\cdot}8^{c,d}$	$11{\cdot}71^{c,d}$	$7{\cdot}47^{a}$	$8{\cdot}76^{b,c}$	$6{\cdot}03^{c}$
9	$30{\cdot}6^{b,c}$	$0{\cdot}433^{b}$	$21{\cdot}9^{b}$	$14{\cdot}72^{d}$	$11{\cdot}31^{c,d}$	$6{\cdot}90^{b}$	$3{\cdot}63^{a,b}$
10	$44{\cdot}9^{e}$	$0{\cdot}590^{e,f,g}$	$33{\cdot}0^{c,d}$	$6{\cdot}68^{a,b}$	$7{\cdot}25^{a}$	$9{\cdot}02^{b,c}$	$8{\cdot}65^{d}$

[A] Results are means for six ewes. [B] Results are means for three ewes (second replicate experiment only).

density (micrograms of DNA per milligram of tissue) diminished to about 60% of the control value ($P < 0{\cdot}01$), but oestradiol caused almost no change in alkaline phosphatase activity (Table 2).

Effects of Progesterone (Groups 3 and 4 v. Group 1)

Giving progesterone for 3 days had little effect on uterine weight, whereas when given for 9 days progesterone increased the weight of the uterus almost as much as did oestradiol in group 2 ($P < 0{\cdot}01$). Compared with the responses to oestradiol, both progesterone treatments caused similar increases in the rate of protein synthesis (group 3 *v.* group 1, $P < 0{\cdot}01$; group 4 *v.* group 1, $P < 0{\cdot}05$), but smaller changes in RNA : DNA ratios ($P < 0{\cdot}01$) and mean cell density ($P < 0{\cdot}01$) in the endometrium and in cytosol concentration of oestradiol receptor ($P < 0{\cdot}01$) in whole uterus. Progesterone caused only a small and insignificant increase in progesterone receptor levels, but a three- to four-fold increase in alkaline phosphatase activity ($P < 0{\cdot}01$).

Effects of Administering Progesterone before and/or together with Oestradiol (Groups 5, 6, 8 and 10 v. Group 2)

Giving progesterone concurrently did not diminish the uterine weight response to oestradiol (group 5 *v.* group 2). When progesterone was given before oestradiol the weight of the uterus on day 14 was further increased, but the effect was significant only in one group of ewes (group 10 *v.* group 2, $P < 0{\cdot}05$). The increase in RNA : DNA ratio elicited by oestradiol was scarcely affected by the administration of progesterone, suggesting either that the mean cell content of RNA in the endometrium had reached near maximal values in ewes in group 2, or that the effects of oestradiol and progesterone were not additive. Progesterone appeared to increase further the rate of protein synthesis in ewes which received oestradiol on days 10–12, but these increases were not significant. There were significant increases in alkaline phosphatase activity in two groups (group 5 *v.* group 2, $P < 0{\cdot}05$; group 8 *v.* group 2, $P < 0{\cdot}01$) that were presumably attributable to progesterone *per se*. The increases in groups 6 and 10 were much smaller, probably because a period of 5 days elapsed between the last injection of progesterone and the time of killing. Giving progesterone further reduced DNA : wet weight ratios in the endometrium, although the decreases were significant only when progesterone was given for 9 days (groups 8 and 10 *v.* group 2, $P < 0{\cdot}01$). The concentration of oestradiol receptor in ewes which received oestradiol on days 10–12 was not changed by giving progesterone for 3 days (groups 5 and 6), but was significantly reduced by about 25% in groups which received progesterone for 9 days (groups 8 and 10 *v.* group 2, $P < 0{\cdot}05$). In the case of the progesterone receptor, administering progesterone significantly decreased the response to oestradiol given on days 10–12 (groups 5, 8 and 10 *v.* group 2, $P < 0{\cdot}01$).

Effects of Administering Oestradiol before Progesterone (Group 7 v. Group 3, and Group 9 v. Group 4)

When oestradiol was given on days 7–9 or 1–3 to ewes receiving progesterone the mean endometrial cell content of RNA on day 14 did not change significantly (group 7 *v.* group 3, group 9 *v.* group 4). Similarly priming with oestradiol at these times had no significant effect on rate of synthesis of protein or alkaline phosphatase activity. Giving oestradiol on days 7–9 did further decrease endometrial cell density

(group 7 *v.* group 3, $P < 0{\cdot}01$), but when given on days 1–3 oestradiol had no such effect. In the case of the hormone receptor concentration on day 14 these prior oestradiol treatments had only small, insignificant effects on the oestrogen receptor and almost no effect on the progesterone receptor.

Results for the recovery of protein from uterine flushings and for the metabolism of [^{3}H]oestradiol and [^{3}H]progesterone by uterine minces are not shown. Several uterine flushings were cloudy and two were visibly contaminated with blood. Most contained between 0·5 and 4·0 mg of protein, but a few contained more than 10 mg. The amount of protein varied widely within treatment groups, and none of the differences between treatment means was significant. When [^{3}H]oestradiol was incubated in uterine minces $97{\cdot}3 \pm 0{\cdot}6\%$ of the recovered radioactivity was associated with oestradiol, the residue being associated with oestrone. In the case of incubations with [^{3}H]progesterone, $95{\cdot}0 \pm 0{\cdot}6\%$ of the recovered radioactivity was associated with progesterone, the residue being associated with a polar metabolite which remained at the origin (Miller *et al.* 1977*a*). With both steroids there were no significant differences between treatments.

Discussion

Both oestradiol and progesterone stimulate RNA and protein synthesis in the endometrium of the ewe (Brinsfield and Hawk 1974; Miller 1976; Miller *et al.* 1977*b*). In this species progesterone does not appear to block oestrogen-induced increases in RNA and protein (Miller 1976) as it does in laboratory rodents (Bronson and Hamilton 1972; Trams *et al.* 1973). Progesterone increased the mean cell content of RNA and rate of synthesis of protein in the endometrium in the present study. The administration of progesterone in several different ways did not reduce the large increase in RNA : DNA ratio elicited by giving oestradiol for 3 days (groups 5, 6, 8 and 10 *v.* group 2); and in the case of protein synthesis these progesterone treatments either did not alter or increased the response to oestradiol. Thus the results clearly support the suggestion (Miller 1976) that the uterus of the ewe is basically different from that of the rat or mouse, inasmuch as progesterone cannot suppress the effects of maximally stimulating doses of oestrogen on RNA and protein synthesis which culminate in large increases in uterine weight.

The changes elicited by the different treatments in the amount of DNA per milligram of endometrium appear more or less inversely related to the changes in RNA : DNA ratio, suggesting that the degree of cellular hypertrophy was the principal determinant of the tissue DNA concentration. However, priming with progesterone did further decrease DNA concentration in ewes which received oestradiol (groups 6 and 10 *v.* group 2), whereas this priming progesterone did not further increase RNA : DNA ratios. Others factors in addition to stromal cell hypertrophy (Miller 1976) probably affect endometrial DNA concentration. Changes in extracellular tissue space related to hormone effects on blood flow and capillary permeability likely occurred, but the contribution of each of these factors in determining the DNA : wet weight ratio is not known.

We sought to simplify the interpretation of our results for cytosol receptor levels in whole uterus by allowing a period of 48 h to elapse between the last hormone injections and the time of killing, thus avoiding short-term 'depletion' effects due to the translocation of cytosol receptors to the nucleus (Jensen *et al.* 1974). The observed

stimulatory effects of oestradiol on the levels of both receptors in the ewe are entirely consistent with reports of oestrogen effects on receptor levels in several other species (see Brenner and West 1975 for a review). Progesterone reduces by 50% or more the cytosol level of oestrogen receptor in oestrogen-treated rats, by interfering with the replenishment or *de novo* synthesis of receptor (Hsueh *et al.* 1976; Pavlik and Coulson 1976). In the present study progesterone did not change oestradiol receptor levels when given together with oestradiol over 3 days (group 5 *v.* group 2), although the response to oestradiol was slightly depressed when progesterone was also given for 6 or more days before the oestrogen treatment. Progesterone alone, however, almost doubled the oestradiol receptor level. A stimulatory effect of progesterone *per se* on the rat endometrial but not myometrial oestradiol receptor has been reported (Mester *et al.* 1974), but we have found no difference in the response of the sheep endometrial and myometrial receptor to changing steroid secretion (Miller *et al.* 1977*a*). It appears that the effects of progesterone on the 'metabolism' of the oestrogen receptor are substantially different in the ewe and the rat, and this may account for the different effects of progesterone on oestrogen-induced RNA and protein synthesis in the uteri of these two species.

The stimulatory effect of oestradiol on progesterone receptor levels was partially blocked by also giving progesterone, especially when the latter hormone was given for 9 days. This may reflect an increase in the rate of inactivation of the progesterone receptor and/or an inhibition of oestradiol-induced receptor synthesis (Milgrom *et al.* 1973; Freifeld *et al.* 1974).

Our results support the finding (Murdoch and White 1968*a*, 1968*b*) that alkaline phosphatase activity in the endometrium of the ewe is regulated by progesterone and, unlike in the case of several laboratory animals, is not influenced by oestrogens. The results show also that large increases in this enzyme activity as well as other progesterone-induced responses such as increased rates of protein synthesis are not associated with any significant elevation of progesterone receptor levels. This may indicate only that, provided they are maintained during hormone treatment, the levels of cytosol receptor found in control ewes are sufficient to mediate these uterine responses. However, it is also possible that this receptor does not have any important role in the induction of these uterine responses to progesterone.

These results for the ovariectomized ewe permit further interpretation of data that we have previously reported for RNA and protein metabolism and hormone receptor levels in the uterus during the oestrous cycle (Miller *et al.* 1977*a*). Oestrus was designated day 0. The high rates of protein synthesis, RNA : DNA ratios and oestrogen receptor levels observed in the endometrium on days 0–2 resulted presumably from high levels of ovarian oestrogen secretion during days −2, −1 and 0. The decline in these parameters from day 5 onwards must have reflected reduced oestrogen stimulation, rather than any inhibitory influence of increasing plasma levels of progesterone. Further, the present results suggest that oestrogen receptor levels and metabolic activity would have declined to even lower levels on days 10–14 in the absence of luteal-phase progesterone. However, in view of the present results, the failure of luteal-phase progesterone to increase the oestradiol receptor level on days 10–14 is puzzling. In the case of the progesterone receptor, high levels on days 0–2 were probably also due to oestrogen secretion on days −2, −1, and 0, but increasing plasma progesterone levels no doubt contributed to the decline in receptor level between days 5 and 14 by hastening the inactivation of available receptor.

The amounts of protein recovered in uterine flushings were similar to those obtained during the first 13 days of pregnancy in the ewe (Roberts *et al.* 1976). In further treatment groups (B. G. Miller, unpublished data) amounts of protein ranging between 2·0 and 4·0 mg were recovered from ovariectomized ewes which had received 12 mg progesterone daily for 18 days. This suggests that the considerable increase in luminal protein that occurs in the ewe after day 14 of pregnancy (Roberts *et al.* 1976) is elicited by the foetus rather than by ovarian progesterone. The ewe seems to differ from the gilt in this respect, since daily administration of progesterone to ovariectomized gilts for 10 or more days causes a striking increase in the protein content of uterine flushings (Knight *et al.* 1973, 1974).

The low level of uterine metabolism of both steroids and the lack of response to a wide range of steroid regimes confirms previous reports (Miller *et al.* 1977*a*; Murphy *et al.* 1977) and other studies using a range of buffers with and without cofactor supplementation (L. Murphy and G. M. Stone, unpublished data). Why the uterus of this species, in contrast to others, should show so little metabolism is not clear.

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