Hormonal regulation of oestrogen and progesterone receptors in cultured bovine endometrial cells

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Changes in the number of progesterone and oestradiol receptors in the endometrium are thought to play a role in the induction of luteolysis. The effect of oestradiol and progesterone on the regulation of their receptors in cultured bovine uterine epithelial and stromal cells was examined to determine the mechanisms involved in this process. Cells were obtained from cows at days 1–3 of the oestrous cycle and were cultured for 4 or 8 days in medium alone (RPMI medium + 5% (v/v) charcoal–dextran stripped newborn calf serum) or with oestradiol, progesterone or oestradiol and progesterone. At the end of culture, receptor binding was measured by saturation analysis. Specific binding of both [³H]ORG 2058 (16 α -ethyl-21-hydroxy-19-nor (6,7-³H) pregn-4-ene-3,20-dione) and [³H]oestradiol to epithelial and stromal cells showed high affinities ($K_d = 1.1 \times 10^{-9}$ and 6×10^{-10} mol l⁻¹, respectively, for progesterone receptors; $K_d = 5.5 \times 10^{-9}$ and 7×10^{-10} mol l⁻¹, respectively, for oestradiol receptors). In the stromal cells, oestradiol (0.1–10 nmol l⁻¹) increased the number of oestradiol receptors from 0.21 ± 0.06 to 0.70 ± 0.058 fmol μ g⁻¹ DNA and the number of progesterone receptors from 1.4 ± 0.83 to 6.6 ± 0.70 fmol μ g⁻¹ DNA in a dose-dependent manner after 4 days of culture ($P < 0.01$). After culture for 8 days, the stimulatory effect of oestradiol increased. Progesterone (50 nmol l⁻¹) had no effect on the number of oestradiol or progesterone receptors ($P > 0.05$). However, progesterone inhibited the stimulatory effect of oestradiol. In epithelial cells, the lower concentrations of oestradiol (0.1 and 1 nmol l⁻¹) stimulated the number of progesterone receptors ($P = 0.05$) after 4 days culture, whereas the highest concentration of oestradiol (10 nmol l⁻¹), progesterone (50 nmol l⁻¹) and progesterone (50 nmol l⁻¹) plus oestradiol (1 nmol l⁻¹) had no effect. After culture for 8 days, the stimulatory effect of oestradiol decreased. In contrast to progesterone receptors, the number of oestradiol receptors increased with oestradiol concentration ($P < 0.01$). These data show that the number of progesterone receptors was higher in the stromal cells than in epithelial cells, whereas the number of oestradiol receptors was higher in the epithelial cells than in stromal cells. Oestradiol upregulates its own receptor and increases the number of progesterone receptors in both cell types *in vitro*, whereas progesterone has little effect, but inhibits the effects of oestradiol on progesterone receptors.

Introduction

The bovine endometrium is a highly sensitive target organ for ovarian steroid hormones (Boos *et al.*, 1996). In ruminants, the uterine epithelium, stroma and myometrium all contain receptors for progesterone and oestrogen (Wathes and Hamon, 1993; Boos *et al.*, 1996). The number of endometrial receptors determines the sensitivity of the uterus to steroid stimulation and the receptors are themselves regulated by steroid hormones. The number of oestradiol and progesterone receptors changes throughout the oestrous cycle in response to changes in circulating steroid hormone concentrations (Boos *et*

al., 1996). Receptors are present in the highest concentrations at postpartum anoestrus, oestrus and during the follicular phase of the oestrous cycle, when plasma progesterone concentrations are low (Vesanan *et al.*, 1988; Vesanan *et al.*, 1991). During the luteal phase of the oestrous cycle, when progesterone production by the corpus luteum increases, oestradiol and progesterone receptor concentrations decrease in the endometrium (Findlay *et al.*, 1982; Zelinski *et al.*, 1982; Vesanan *et al.*, 1988; Vesanan *et al.*, 1991; Salamonsen *et al.*, 1992; Boos *et al.*, 1996). It is generally accepted that progesterone and oestradiol exert opposing effects on steroid receptors in the uterus. Oestradiol increases the number of oestradiol and progesterone receptors and progesterone decreases the number of these receptors (Katzenellenbogen, 1980; Leavitt *et al.*, 1983; Clark *et al.*, 1985).

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The timing and initiation of luteolysis are critical for the normal oestrous cycle in ruminant animals (Lamming and Mann, 1995). Changes in the number of progesterone and oestradiol receptors are thought to play a role in luteolysis in sheep and cattle (McCracken *et al.*, 1984; Spencer *et al.*, 1995; Wathes and Lamming, 1995). The number of uterine progesterone and oestradiol receptors may influence uterine PGF_{2α} secretion, and thus the lifespan of the corpus luteum during the oestrous cycle (Zollers *et al.*, 1993) and early pregnancy (Spencer and Bazer, 1995). The duration of the luteal phase is controlled by the time of initiation and duration of exposure to progesterone. Early administration of progesterone shortens the interoestrus interval in ewes and cows, and blocking progesterone stimulation using a progesterone receptor antagonist delays luteolysis in sheep (Woody and Ginther, 1968; Ginther, 1970; Ottobre *et al.*, 1980; Garrett *et al.*, 1988). Owing to the inhibitory effect of progesterone on progesterone and oestradiol receptors, extended exposure of the uterus to progesterone downregulates progesterone receptors and desensitizes the uterus to progesterone. As the inhibitory effect of progesterone decreases, the number of oestradiol receptors increases in the presence of oestrogen and thus increases the sensitivity of the uterus to oestrogen. In addition, the number of oxytocin receptors in the endometrium is regulated by progesterone and oestrogen; progesterone inhibits oxytocin receptors and oestrogen enhances oxytocin receptors (Hixon and Flint, 1987; Spencer *et al.*, 1995; Spencer *et al.*, 1996). Therefore, downregulation of progesterone increases oxytocin receptors and the sensitivity of the endometrium to oxytocin stimulation, which induces luteolytic pulsatile release of PGF_{2α} (McCracken *et al.*, 1984).

The various cell types present in the bovine endometrium exhibit significantly different patterns of oestrogen and progesterone receptor immunoreactivities during the oestrous cycle (Boos *et al.*, 1996). In sheep, oestradiol receptor distribution varies according to the cell type, tissue compartment (caruncular or intercaruncular endometrium) and the location of the particular cell type within that compartment (Salamonsen *et al.*, 1992; Spencer *et al.*, 1996). This suggests that different cell types within the uterus respond differently to identical hormonal stimuli (Boos *et al.*, 1996).

Modulation of progesterone and oestrogen receptors by steroid hormones in different endometrial cell populations is not completely understood due to the complexity of the interactions involved. In this study, endometrial stromal and epithelial cells in primary culture were used to investigate hormonal regulation of oestradiol and progesterone receptors in the bovine uterus.

Materials and Methods

Chemicals and reagents

Tissue culture medium (RPMI 1640), Hank's buffered saline solution (HBSS, calcium and magnesium free), newborn calf serum (NBCS), antibiotics and trypan blue were purchased from GIBCO (Grand Island, NY). Collagenase

(Type II), trypsin (Type III, from bovine pancreas), DNase I (Type I, from bovine pancreas), gentamicin, calf thymus DNA, Hoechst No. 33258, BSA, oestradiol and progesterone were purchased from Sigma Chemical Co. (St Louis, MO). Stock solutions of oestradiol and progesterone were prepared by dissolving the steroids in ethanol. Matrigel was obtained from VWR Canlab (Ontario). Bio-Rad protein assay-dye reagent concentrate was obtained from Bio-Rad Laboratories (Hercules, CA). [2, 4, 6, 7-³H] oestradiol (83.0 Ci mmol⁻¹), [³H]ORG 2058 (16α-ethyl-21-hydroxy-19-nor [6,7-³H] pregn-4-ene-3,20-dione; 49.0 Ci mmol⁻¹) and unlabelled ORG were purchased from Amersham Life Science (Oakville, Ontario). Diethylstilboestrol was obtained from ICN Biochemicals Inc. (Aurora, OH).

Preparation and culture of cells

Uteri from cows at days 1–3 of the oestrous cycle (ovaries with a corpus haemorrhagicum) were collected at an abattoir and transported on ice to the laboratory. The presence of a corpus haemorrhagicum in the experimental tissue allowed accurate estimation of the stage of the oestrous cycle, thus reducing variability among experiments. In addition, the tissue was taken before exposure to high concentrations of progesterone, allowing the long-term effects of progesterone to be studied *in vitro*. Endometrial epithelial and stromal cells were separated by modifying the procedure of Fortier *et al.* (1988). Briefly, the two uterine horns were placed in sterile HBSS containing 100 iu penicillin, 100 µg streptomycin ml⁻¹ and 0.25 µg amphotericin ml⁻¹. The myometrial layers were dissected and each uterine horn was then everted to expose the epithelium. The everted horns were first digested for 2 h in HBSS with 0.3% (w/v) trypsin at room temperature to obtain epithelial cells. At the end of the incubation, the digested horns were scraped lightly with forceps, and washed twice in HBSS. The tissue was further digested to obtain stromal cells by incubating in HBSS with 0.064% (w/v) trypsin III, 0.064% (w/v) collagenase II and 0.032% (w/v) DNase I for 45 min at 37°C. Immediately after each cell suspension was collected, 10% NBCS was added to inhibit the trypsin. For epithelial cells, the cell suspension was centrifuged at 60 g for 5 min and the pellet was then washed three times with HBSS. For further purification, the epithelial cell pellet was suspended in 20 ml RPMI medium supplemented with 5% NBCS and 50 µg gentamicin ml⁻¹, plated onto 100 mm × 20 mm Nunclon Petri dishes (Grand Island, NY) and then incubated at 37°C with 5% CO₂ and 95% air for 3 h. At the end of incubation, contaminating stromal cells adhered to the dish and the floating epithelial cells were collected. After cell counting and viability determination by trypan blue exclusion, 2.5 × 10⁶ cells per dish were plated onto Matrigel-coated 100 mm × 20 mm dishes. Since the epithelial cells only attached to the plates after approximately 48 h, they were cultured for a further 4 days at 37°C in humidified air (5% CO₂) in RPMI-1640 medium containing 5% NBCS depleted of steroids by dextran–charcoal extraction. The cells were then cultured for 4 or 8 days in the presence or absence of various concentrations of oestrogen and progesterone. The medium was changed every 2 days.

For stromal cells, the cell suspension was centrifuged at

60 g for 5 min to remove clumps of cells and then the supernatant was centrifuged at 1000 g for 10 min. The pelleted cells were washed twice with HBSS. The stromal cell suspension was plated onto dishes at a concentration of 1×10^7 cells per dish and after a 3 h incubation, the floating cells were washed away by gentle pipetting. The attached stromal cells were then cultured in RPMI-1640 medium containing 5% NBCS depleted of steroids by dextran-charcoal extraction, in the presence or absence of various concentrations of oestrogen and progesterone at 37°C in humidified air (5% CO₂) for 4 or 8 days. The medium was changed every 2 days.

The homogeneity of the cell populations was examined by immunocytochemistry. Epithelial cell contamination of stromal cells was about 3% and stromal cell contamination of epithelial cells was less than 1% (Xiao and Goff, 1998). The cells were approximately 40% confluent at the time of treatment and were 70–80% and 100% confluent on days 4 and 8, respectively.

Progesterone and oestrogen receptor assays

After treatment with different hormonal regimens for 4 or 8 days, uterine cells were rinsed with HBSS and detached from the Petri dishes with HBSS containing 1 mmol EDTA l⁻¹ and gentle scraping with a rubber scraper. The cells were pelleted at 500 g for 5 min and resuspended in 250 µl HED buffer (20 mmol Hepes l⁻¹, 1.5 mmol EDTA l⁻¹, 0.25 mmol dithiothreitol l⁻¹, pH 7.4). After removal of 5 µl cell suspension for DNA measurement, the remaining cell suspension was sonicated and pelleted at 15000 g for 10 min. The supernatant was collected and the pellet was treated twice with 150 µl extraction buffer (0.6 mol KCl l⁻¹ in HED) on ice for 20 min. This treatment extracted any remaining nuclear material. At the end of the extraction, the cell debris was separated by centrifugation at 15000 g for 10 min. The supernatant was combined with the initial supernatant collected after sonication and was used for oestrogen and progesterone receptor measurements. The protein concentration was determined using the method of Bradford (1976) (Bio-Rad Laboratories, CA). The DNA content was measured by the bisbenzimidazole fluorescent dye method of Labarca and Paigen (1980). Calf thymus DNA was used as the standard at concentrations of 4, 8, 16, 32, 64, 128 and 250 ng ml⁻¹.

Saturation analysis of oestradiol and progesterone receptors in primary cultures of uterine endometrial cells was performed in the presence of 1 nmol oestradiol l⁻¹ for 4 days. The supernatants were incubated with 0.5–20 nmol [³H]ORG 2058 l⁻¹ or 0.5–10 nmol [³H]oestradiol l⁻¹ for progesterone and oestradiol receptor measurements, respectively. The binding affinities of [³H]ORG 2058 and [³H]oestradiol for cell supernatants were determined using the least squares curve fitting program LIGAND (Munson and Rodbard, 1980). ORG 2058 was used for measurement of progesterone receptors because of its low non-specific binding. Furthermore, in contrast to progesterone, it does not bind to the corticosteroid receptor. The data are presented as saturation and Scatchard curves (Scatchard, 1949).

Supernatants (0.8–1.0 mg ml⁻¹ total protein) were incubated with 10 nmol [³H]ORG 2058 l⁻¹ in HED buffer with or without

a 200-fold excess of unlabelled ORG 2058 at 4°C for 2.5–4.0 h to measure total and non-specific binding, respectively, and to determine the effect of steroid treatment on the number of progesterone receptors. For the oestradiol receptor assay, cell supernatants were incubated with 5 nmol [³H]oestradiol l⁻¹ in HED buffer with or without a 200-fold excess of unlabelled diethylstilboestrol at 4°C for 16 h, and then incubated at 30°C for 3 h. After incubation, bound and free steroid were separated by adsorption with dextran-coated charcoal (DCC; 0.5% (w/v) charcoal and 0.05% (w/v) dextran in HED buffer) for 15 min at 4°C as described by Leake and Habib (1987). Specific binding (total minus non-specific binding) values are expressed as femtomole per microgram of DNA.

Statistical analysis

Each treatment was carried out in triplicate using the cells from one uterus and each experiment was repeated with six different uteri. The data were log transformed where necessary to eliminate heterogeneity of variance. For each cell type, regression analysis was used to describe the effect of dose of oestradiol on the number of receptors, and a 2 × 2 factorial design (ANOVA) was used to analyse the effects of oestradiol, progesterone and the duration of culture. The ANOVA included the main effects of duration of culture, progesterone treatment, oestradiol treatment and all interactions. Since uterus was nested within an experiment it was included as a random variable in the *F* test for the effect of experiment. Simple effect comparisons to determine differences between individual means were performed using preplanned orthogonal contrasts. A probability of *P* < 0.05 was considered to be statistically significant. The data were analysed using the computer program JMP (SAS Institute Inc., NC).

Results

Characterization of oestradiol and progesterone receptor binding in endometrial cells

Specific binding of [³H]ORG 2058 and [³H]oestradiol by epithelial and stromal cells from bovine uterine endometrium collected at days 1–3 of the oestrous cycle was saturable and showed high affinities (Figs 1 and 2). The progesterone receptors were saturated at 5 and 10 nmol l⁻¹ for epithelial and stromal cells, respectively (Fig. 1a,c). The dissociation constants (*K_d*) were 1.1 nmol l⁻¹ for epithelial cells (Fig. 1b) and 0.6 nmol l⁻¹ for stromal cells (Fig. 1d). The oestradiol receptors were saturated at 5 nmol l⁻¹ for both epithelial and stromal cells (Fig. 2a,c). The dissociation constants (*K_d*) were 5.5 nmol l⁻¹ for epithelial cells (Fig. 2b) and 0.7 nmol l⁻¹ for stromal cells (Fig. 2d).

Effect of oestradiol and progesterone on the number of progesterone receptors in stromal cells

Oestradiol stimulated the number of progesterone receptors in stromal cells after 4 days of incubation in a linear

Fig. 1. Saturation analysis of specifically bound $[^3\text{H}]\text{ORG}$ 2058 (16 α -ethyl-21-hydroxy-19-nor (6,7- ^3H) pregn-4-ene-3,20-dione) to primary cultures of bovine uterine cells. Cells were cultured in RPMI 1640 medium containing 5% (v/v) newborn calf serum in the presence of 1 nmol oestradiol l^{-1} for 4 days. The whole cell extracts were treated with $0.5\text{--}20 \times 10^{-9}$ mol $[^3\text{H}]\text{ORG}$ 2058 l^{-1} with or without a 200-fold molar excess of unlabelled ORG 2058. Total binding ($\square$), non-specific binding ($\blacklozenge$) and specific binding ($\blacksquare$) by (a) epithelial cells and (c) stromal cells are presented as saturation curves. Corresponding Scatchard plots of specific binding are presented in (b) ($n = 0.65$ fm μg^{-1} DNA; $K_d = 1.1 \times 10^{-9}$ mol l^{-1}) and (d) ($n = 3.75$ fm μg^{-1} DNA; $K_d = 6 \times 10^{-10}$ mol l^{-1}). B:F, ratio of bound:free.

dose-dependent manner ($P < 0.05$, $r^2 = 0.12$), with the maximum effect at 10 nmol l^{-1} (Fig. 3a). The time-dependent effect of oestrogen and progesterone on the number of progesterone receptors in stromal cells is also shown (Fig. 3b). ANOVA showed an effect of oestradiol (1 nmol l^{-1}) and duration of culture on the number of progesterone receptors in stromal cells ($P < 0.01$). There was a duration of culture $\times$ oestradiol interaction ($P < 0.05$), showing that the response to oestradiol increased with time, and a progesterone $\times$ oestradiol interaction ($P < 0.001$) showing that progesterone

had no effect but that it inhibited the oestradiol-induced increase in progesterone receptors.

Effect of oestradiol and progesterone on the number of progesterone receptors in epithelial cells

Oestradiol stimulated the number of progesterone receptors in epithelial cells after 4 days (Fig. 4a). Regression analysis revealed that the best fit regression line was cubic

Fig. 2. Saturation analysis of specifically bound [³H]oestradiol to primary cultures of bovine uterine cells. Cells were cultured in RPMI 1640 medium containing 5% (v/v) new born calf serum in the presence of 1 nmol oestradiol l⁻¹ for 4 days. The whole cell extracts were treated with 0.5–10 × 10⁻⁹ mol [³H]oestradiol l⁻¹ with or without a 200-fold molar excess of unlabelled diethylstilboestrol. Total binding (□), non-specific binding (◆) and specific binding (■) by (a) epithelial cells and (c) stromal cells are presented as saturation curves. Corresponding Scatchard plots of specific binding are presented in (b) ($n = 2.1 \text{ fmol } \mu\text{g}^{-1} \text{ DNA}$; $K_d = 5.5 \times 10^{-9} \text{ mol l}^{-1}$) and (d) ($n = 0.45 \text{ fmol } \mu\text{g}^{-1} \text{ DNA}$; $K_d = 7 \times 10^{-10} \text{ mol l}^{-1}$). B:F, ratio of bound:free.

($P < 0.05$, $r^2 = 0.3$). The lowest concentration of oestradiol used (0.1 nmol l⁻¹) was the most effective (167% higher than the control). Progesterone had no effect on the number of progesterone receptors in cultured epithelial cells (Fig. 4b). ANOVA showed an effect of oestradiol ($P = 0.05$) and an oestradiol × progesterone interaction ($P < 0.05$). Progesterone blocked the oestradiol-induced increase in progesterone receptors at 4 days of culture, and there was no effect of either progesterone or oestradiol after 8 days of culture.

Effect of oestradiol and progesterone on the number of oestradiol receptors in stromal cells

Oestradiol stimulated the number of oestradiol receptors in stromal cells after 4 days ($P < 0.01$) (Fig. 5a). The effect of oestradiol was dose dependent; a regression analysis showed that the best fit regression line was quadratic ($P < 0.001$, $r^2 = 0.22$). The maximum response was observed at 1 nmol l⁻¹, after which the response decreased. The time-

Fig. 3. Time- and dose-dependent effect of oestradiol and progesterone on the number of progesterone receptors in bovine endometrial stromal cells. (a) Primary stromal cells were cultured in RPMI 1640 medium containing 5% (v/v) newborn calf serum (NBCS) in the presence or absence of oestradiol (0.1, 1.0 or 10 nmol l⁻¹) for 4 days. (b) Primary stromal cells were cultured in RPMI 1640 medium containing 5% (v/v) NBCS in the presence or absence of 1 nmol oestradiol l⁻¹, 50 nmol progesterone l⁻¹, or 50 nmol progesterone l⁻¹ plus 1 nmol oestradiol l⁻¹ for (□) 4 or (■) 8 days. Data represent least square means $\pm$ SE; the minimum detection limit is 0.11 fmol µg⁻¹ DNA.

dependent effect of oestradiol and progesterone on the number of oestradiol receptors in stromal cells is also shown (Fig. 5b). Progesterone had no effect on the number of oestradiol receptors. Effects of oestradiol treatment and duration of culture were detected ($P < 0.001$), and there was a significant progesterone $\times$ oestradiol interaction ($P < 0.05$). A duration $\times$ progesterone $\times$ oestradiol interaction was suggested ($P = 0.08$). The number of oestradiol receptors increased markedly in stromal cells between days 4 and 8 of culture ($P > 0.001$). Progesterone had an inhibitory effect on the stimulation of oestradiol receptors by oestradiol after 4 days of culture ($P < 0.05$), but not after 8 days.

Effect of oestradiol and progesterone on the number of oestradiol receptors in epithelial cells

Oestradiol stimulated the number of oestradiol receptors in epithelial cells in a dose-dependent manner after 4 days

Fig. 4. Time- and dose-dependent effect of oestradiol and progesterone on the number of progesterone receptors in bovine endometrial epithelial cells. (a) Primary epithelial cells were cultured in RPMI 1640 medium containing 5% (v/v) newborn calf serum (NBCS) in the presence or absence of oestradiol (0.1, 1.0 or 10 nmol l⁻¹) for 4 days. (b) Primary epithelial cells were cultured in RPMI 1640 medium containing 5% (v/v) NBCS in the presence or absence of 1 nmol oestradiol l⁻¹, 50 nmol progesterone l⁻¹, or 50 nmol progesterone l⁻¹ plus 1 nmol oestradiol l⁻¹ for (□) 4 or (■) 8 days. Data represent least square means $\pm$ SE; the minimum detection limit is 0.11 fmol µg⁻¹ DNA.

(Fig. 6a). Regression analysis showed that the best fit regression line was linear ($P < 0.005$, $r^2 = 0.51$). The lowest concentration of oestradiol (0.1 nmol l⁻¹) had no effect on the number of oestradiol receptors in epithelial cells ($P > 0.05$). The number of receptors did not respond to progesterone (50 nmol l⁻¹) (Fig. 6b) and there was no change in the response to oestradiol between 4 and 8 days of culture.

Discussion

The mechanisms involved in the timing of luteolysis in cattle are not completely understood. Changes in steroid receptors are considered to play an important role in this process (Meyer *et al.*, 1988; Zollers *et al.*, 1993). A culture system in which the cells respond in a similar manner to that observed *in vivo* is required to investigate the mechanisms involved in luteolysis at the cellular level (Katzenellenbogen, 1980;

Fig. 5. Time- and dose-dependent effect of oestradiol and progesterone on the number of oestrogen receptors in bovine endometrial stromal cells. (a) Primary stromal cells were cultured in RPMI 1640 medium containing 5% (v/v) newborn calf serum (NBCS) in the presence or absence of oestradiol (0.1, 1 or 10 nmol l⁻¹) for 4 days. (b) Primary stromal cells were cultured in RPMI 1640 medium containing 5% (v/v) NBCS in the presence or absence of 1 nmol oestradiol l⁻¹, 50 nmol progesterone l⁻¹, or 50 nmol progesterone l⁻¹ plus 1 nmol oestradiol l⁻¹ for (□) 4 or (■) 8 days. The number of receptors is presented on a logarithmic scale in (b). Data represent least square means $\pm$ SE; the minimum detection limit is 0.06 fmol µg⁻¹ DNA.

Leavitt *et al.*, 1983; Clark *et al.*, 1985). In this study, a primary cell culture system was used to investigate the effects of steroid hormones on the number of oestrogen and progesterone receptors in epithelial and stromal cells of the bovine endometrium.

Low-speed cell supernatants were used for receptor measurements in this study because in preliminary experiments, no differences were observed in the number of receptors in supernatants separated by ultra-speed or low-speed centrifugation. This was also demonstrated by Leake and Habib (1987). The results show that both progesterone and oestrogen receptors in cultured bovine endometrial cells are of high affinity ($K_d = 1.1$ and 0.6 nmol l⁻¹, respectively, for progesterone receptors of epithelial and stromal cells; $K_d = 5.5$ and 0.7 nmol l⁻¹, respectively, for oestradiol receptors of epithelial and stromal cells). These values are similar to the affinities of oestradiol and progesterone receptors in

Fig. 6. Time- and dose-dependent effect of oestradiol and progesterone on the number of oestrogen receptors in bovine endometrial epithelial cells. (a) Primary epithelial cells were cultured in RPMI 1640 medium containing 5% (v/v) newborn calf serum (NBCS) in the presence or absence of oestradiol (0.1, 1 or 10 nmol l⁻¹) for 4 days. (b) Primary epithelial cells were cultured in RPMI 1640 medium containing 5% (v/v) NBCS in the presence or absence of 1 nmol oestradiol l⁻¹, 50 nmol progesterone l⁻¹, or 50 nmol progesterone l⁻¹ plus 1 nmol oestradiol l⁻¹ for (□) 4 or (■) 8 days. Data represent least square means $\pm$ SE; the minimum detection limit is 0.06 fmol µg⁻¹ DNA.

endometrial tissue reported by others. Vesanen *et al.* (1988) reported that the K_d values of oestradiol receptors in bovine endometrial tissue at different stages of the oestrous cycle ranged from 0.26 to 2.5 nmol l⁻¹ and that of progesterone receptors from 0.14 to 0.17 nmol l⁻¹. Meyer *et al.* (1988) reported a K_d value of 2.6 nmol l⁻¹ for endometrial cytosolic progesterone receptors in cattle. In cultured uterine epithelial cells of immature rats, the K_d values of cytosolic and nuclear oestradiol receptors were 0.56 and 0.27 nmol l⁻¹, respectively; and those of stromal cells were 0.42 and 0.32 nmol l⁻¹, respectively (McCormack and Glasser, 1980). In the present study, oestradiol and progesterone receptors were detectable in the primary culture system used and their affinities approximate the values reported previously for endometrial tissues.

Two forms have been reported for both the oestradiol and the progesterone receptor. Forms A and B of the progesterone

receptor are present in human endometrium (Tseng and Zhu, 1997). The oestradiol receptor exists in the classical α form and the more recently discovered β form (Kuiper *et al.*, 1996). The β form is present in the bovine endometrium (Rosenfeld *et al.*, 1998) and may be involved in uterine function. The different forms of the oestradiol and progesterone receptors are likely to be present in the cell preparations used in the present study. However, no attempt was made to determine which form of each of the receptors changes in response to steroid treatment.

The basal concentration of progesterone receptors was higher in stromal cells than in epithelial cells. This is in agreement with what is observed in the endometrium during the early luteal phase in cows (Boos *et al.*, 1996). In contrast, the basal concentration of oestradiol receptors was higher in epithelial cells than in stromal cells, whereas in cattle and ovariectomized sheep, the number of oestradiol receptors was higher in the stromal cells (Ing and Tornesi, 1997). This may be related to the stage of the cycle at which the investigation was conducted, since receptor numbers change during the oestrous cycle (Wathes and Hamon, 1993; Boos *et al.*, 1996). In addition, luminal and glandular epithelial cells were not separated in the present study and it is possible that there are differences between these two cell types.

Progesterone and oestradiol receptor concentrations in the endometrium are highest at oestrus and during the follicular phase of the oestrous cycle when endogenous oestradiol is high (Findlay *et al.*, 1982; Zelinski *et al.*, 1982; Vesanan *et al.*, 1988; Vesanan *et al.*, 1991; Salamonsen *et al.*, 1992; Boos *et al.*, 1996). This is probably due to upregulation of these receptors by oestradiol. In ovariectomized ewes, oestradiol treatment increased the number of progesterone and oestradiol receptors in both epithelial and stromal cells (Wathes *et al.*, 1996; Ing and Tornesi, 1997). Oestradiol also induced synthesis of endometrial progesterone receptors *in vitro* and *in vivo* in rats (Kassis *et al.*, 1984) and sheep (Zelinski *et al.*, 1980). In the present study, oestradiol increased progesterone and oestradiol receptor concentrations in a dose- and time-dependent manner in both cell types. This is in agreement with the observations *in vivo*. Although oestradiol has been shown to upregulate progesterone receptors in several cell culture systems, this is the first report of upregulation of oestradiol receptors by oestradiol *in vitro*. Furthermore, the effect of oestradiol was more pronounced in stromal cells than in epithelial cells. This is similar to data from ovariectomized ewes, in which oestradiol treatment caused a greater increase in the number of oestradiol receptors in the stroma than in the luminal epithelium, and the response in the stromal cells appeared earlier and was more prolonged than in the other cell type (Ing and Tornesi, 1997). In the present study, oestradiol stimulation of progesterone and oestradiol receptor concentrations also increased with time in the stromal cells, whereas it diminished during extended culture in the epithelial cells. This is consistent with the observation of Inaba *et al.* (1988) that the number of progesterone receptors in epithelial cells decreases between days 4 and 8 of oestradiol treatment, while that in stromal cells remains high. This suggests that oestradiol stimulation of receptors differs between the two cell types.

In contrast to the stimulatory action of oestradiol, progesterone inhibits both oestradiol and progesterone receptors (Katzenellenbogen, 1980; Leavitt *et al.*, 1983; Clark *et al.*, 1985; Sumida *et al.*, 1988). This inhibitory effect is probably responsible for the decrease in endometrial receptors by the midluteal phase in cows (Meyer *et al.*, 1988) and ewes (Miller *et al.*, 1977). The results of the present study suggest that progesterone is unable to decrease the number of progesterone and oestradiol receptors below the basal concentrations in untreated isolated cells. It is possible that the concentration of progesterone used (close to the highest concentration observed in peripheral plasma) was not high enough to exert an inhibitory effect *in vitro*, or that an interaction between the different cell types is necessary for a normal response. Wathes *et al.* (1996) reported differences between ovariectomized steroid-treated ewes and normal animals; progesterone treatment did not reduce oestradiol receptor concentrations in ovariectomized ewes as was observed in cyclic ewes. It was suggested that ovarian factors other than progesterone and oestradiol are involved in oestradiol receptor regulation. Thus, other factors (such as growth factors and cytokines) may also have a role in receptor regulation in isolated cells.

Although progesterone did not inhibit the number of receptors, it inhibited the stimulatory effect of oestradiol on progesterone receptors in both cell types and on oestradiol receptors in stromal cells. This is in agreement with studies *in vivo* in ovariectomized ewes, in which progesterone inhibited the increase in the number of progesterone receptors induced by oestradiol treatment (Stone *et al.*, 1978; Wathes *et al.*, 1996). The concentration of oestradiol used in the present study was much higher than that in the peripheral plasma. However, the effect of progesterone was still apparent, which suggests that the ratio of progesterone to oestradiol is not important. This is in agreement with a study in guinea-pig fetuses, in which even a large excess of oestradiol did not increase the number of progesterone receptors as long as progesterone was present (Sumida and Pasqualini, 1979). It is not clear why progesterone did not inhibit oestradiol stimulation of oestradiol receptors in epithelial cells. There may be a difference in receptor regulation in the two cell types, or downregulation of oestradiol receptors in epithelial cells may be mediated by the stromal cells as suggested by Wathes *et al.* (1996).

In conclusion, this is the first report of the effect of steroid hormones on the number of progesterone and oestradiol receptors in primary cell cultures of bovine uterine endometrial cells. Oestradiol increased the number of progesterone and oestradiol receptors in both cell types. Progesterone had no effect on the number of receptors, but inhibited the oestradiol-induced increase in progesterone receptors in both cell types. These results support the hypothesis that an interaction among cell types is necessary for the normal function of epithelial cells. Therefore, the isolated cell model system used in this study will be very useful for investigation of the effect of steroid hormones and cell type interactions on endometrial function in cows.

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