The effects of lipopolysaccharide and interleukins-1α, -2 and -6 on oxytocin receptor expression and prostaglandin production in bovine endometrium

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Abstract

Up-regulation of endometrial oxytocin receptor (OTR) expression followed by an increase in pulsatile endometrial prostaglandin (PG) $F_{2\alpha}$ secretion causes luteolysis in cattle. Inhibition of luteolysis is essential for the maternal recognition of pregnancy but also occurs in association with endometritis. The factors regulating OTR expression at this time are unclear. The OTR gene promoter region contains binding elements for acute phase proteins but their function has not been established. This study investigated the effects of various cytokines on OTR expression and on $PGF_{2\alpha}$ and PGE_2 production in explant cultures of bovine endometrium. Endometrium was collected in the late luteal phase (mean day of cycle 15·4 ± 0·50) or early luteolysis (mean day of cycle 16·4 ± 0·24) as determined by the initial concentration of endometrial OTR. Explants were treated for 48 h with: (i) lipopolysaccharide (LPS) and/or dexamethasone (DEX), (ii) ovine interferon-tau (oIFN-τ), or (iii) human recombinant interleukin (IL)-1α, -2 or -6. OTR mRNA was then measured in the explants by *in situ* hybridisation and the medium was collected for measurement of $PGF_{2\alpha}$ and PGE_2 by RIA. LPS treatment stimulated production of $PGF_{2\alpha}$, whereas DEX either alone or in combination with LPS was inhibitory to both $PGF_{2\alpha}$ and PGE_2. Neither of these treatments altered OTR mRNA expression. oIFN-τ reduced OTR mRNA expression but stimulated production of both $PGF_{2\alpha}$ and PGE_2. In endometrial samples collected in the late luteal phase, IL-1α, -2 and -6 all inhibited OTR mRNA expression, but IL-1α and -2 both stimulated $PGF_{2\alpha}$ production. In contrast, when endometrium was collected in early luteolysis, none of the interleukins altered OTR expression or caused a significant stimulation of $PGF_{2\alpha}$ production but IL-2 increased PGE_2. Neither IL-1α nor -2 altered OTR promoter activity in Chinese hamster ovary cells transfected with a bovine OTR promoter/chloramphenicol acetyl transferase reporter gene construct. In conclusion, the action of interleukins on both OTR mRNA expression and endometrial prostaglandin production alters around luteolysis. Pro-inflammatory interleukins suppress OTR expression in the late luteal phase, while LPS stimulates $PGF_{2\alpha}$ without altering OTR mRNA expression. IL-I and -2 and LPS are therefore unlikely to initiate luteolysis but may cause raised production of $PGF_{2\alpha}$ during uterine infection.

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Introduction

Luteal regression in non-pregnant cows involves the action of oxytocin on endometrial oxytocin receptors (OTR) stimulating pulsatile secretion of prostaglandin (PG) $F_{2\alpha}$ by the endometrium (Flint *et al.* 1994, Thatcher *et al.* 1995, Wathes & Lamming 1995). OTR start to develop in the luminal epithelium (LE) between days 15 and 17 days after oestrus (Jenner *et al.* 1991, Robinson *et al.* 1999). The subsequent increase in pulsatile $PGF_{2\alpha}$ on about days 16–17 causes regression of the corpus luteum (Kindahl *et al.* 1976, McCracken *et al.* 1999). In early pregnancy endometrial OTR expression is suppressed by interferon-tau (IFN-τ), which is secreted by the bovine blastocyst from day 8 of gestation, with production peaking on days 17–19 then declining rapidly (Roberts *et al.* 1992). In pregnant cows the episodes of $PGF_{2\alpha}$ still occur but their amplitude is much reduced (Parkinson & Lamming 1990). In the pregnant ewe basal secretion of endometrial $PGF_{2\alpha}$ is higher than in non-pregnant animals (Zarco *et al.* 1988,

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Payne & Lamming 1994), but the pulsatile pattern is lost and luteolysis (LS) is not initiated.

In dairy cows there is a high rate of early embryo mortality. Approximately 25% of bovine embryos are lost before day 16 of gestation and do not, therefore, influence the length of the luteal phase, while a further 7–10% die between days 16 and 60 (Wathes 1992). In addition, uterine infections are prevalent after calving. This can lead to endometritis, which is present in about 10% of UK cows (Sheldon 1999) and which is associated with prolongation of the luteal phase, thus delaying the return to oestrus (Olson *et al.* 1986). Prolonged luteal phases are currently an increasing problem in high yielding dairy cows (Opsomer *et al.* 1998). These conditions cause a significant reduction in fertility and are of considerable economic importance to dairy farmers.

The timing of endometrial OTR development is crucial in determining the length of the oestrous cycle, but it is uncertain how this process is regulated. Oestradiol administered *in vivo* stimulates an increase in uterine OTR in the ewe (Beard & Lamming 1994, Wathes *et al.* 1996) but OTR up-regulation *in vitro* occurs spontaneously in the absence of any oestrogen (Sheldrick *et al.* 1993, Horn *et al.* 1998, Leung & Wathes 2000). Progesterone inhibits the expression of OTR in the early luteal phase, but up-regulation of OTR in the bovine LE in the late luteal phase occurs before any decrease in circulating progesterone and independently of any change in endometrial progesterone receptor expression (Robinson *et al.* 1999). OTR genes have been sequenced in various species: man (Inoue *et al.* 1994); rat Rozen *et al.* (1995); cow (Bathgate *et al.* 1995) and several groups of response elements have been identified in their upstream or intronic regions. Half palindromic oestrogen response elements are present, but their activity in the bovine uterus is uncertain (Horn *et al.* 1998, Leung & Wathes 2000). Acute phase protein binding sites could potentially be controlled by cytokines via cytokine-induced transcription factors such as nuclear factor for interleukin (IL)-6 expression (NF-IL-6), although this has not been demonstrated. These observations suggest that OTR expression is regulated in a complex fashion by a number of different pathways which balance both inhibitory and excitatory mechanisms (Ivell *et al.* 1998).

The preimplantation bovine embryo expresses paternal major histocompatibility complex molecules of class 1 (Templeton *et al.* 1987) but nevertheless normally avoids maternal rejection. This is achieved in part through local production of potential immunomodulators including cytokines, growth factors and prostaglandins (Hansen 1997, Martal *et al.* 1997). In addition, the balance of cytokine production is shifted from a predominant Th1 type response to a Th2 type during pregnancy (Wegmann *et al.* 1993, Hansen 1997). Th1 cells mediate cytotoxicity and delayed type hypersensitivity whereas Th2 cells induce B cell activation. In mice, elevations of type 1 cytokines such as IL-1 and -2 during pregnancy have been associated with fetal resorption (Tangri & Raghupathy 1993), spontaneous abortion (Tangri *et al.* 1994) and premature labour (Romero *et al.* 1991). In women premature labour can also be induced by bacterial infection (Romero *et al.* 1989*a*,*b*). IL-1 mRNA and protein are present in bovine endometrium (Davidson *et al.* 1995, Leung *et al.* 2000) and IL-1 stimulates endometrial production of prostaglandins (Bany & Kennedy 1995, Davidson *et al.* 1995). IL-2 mRNA is down-regulated in early bovine pregnancy (Leung *et al.* 2000).

The aim of this study was, therefore, to determine whether pro-inflammatory interleukins or lipopolysaccharide (LPS, a bacterial product increased during infection) influence the luteolytic process in cows. The effects of various interleukins (IL-1α, -2 and -6) and LPS were tested on endometrial OTR expression and $PGF_{2\alpha}$ and PGE_2 production using endometrial explant cultures. IFN-τ (a known inhibitor of OTR expression) and dexamethasone (DEX) (an inhibitor of prostaglandin synthesis) were used to validate the culture system. The possible effects of IL-1α and -2 on OTR promoter function were also investigated in transfection experiments using Chinese hamster ovary (CHO) cells.

Materials and Methods

Reagents

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

Tissue collection and culture

Holstein/Friesian cows were slaughtered by captive bolt in the luteal phase. Tissue was obtained from 13 cows on the following days of the oestrous cycle: day 12, $n=1$; day 15, $n=3$; day 16, $n=5$ and day 17, $n=4$. The whole uterus was collected from the abattoir, transferred to the laboratory on ice and opened in a laminar flow hood within 2 h of slaughter. A 1 cm cross section of uterine horn was frozen in isopentane in liquid nitrogen for initial estimation of OTR expression by *in situ* hybridisation.

The method of endometrial explant culture is based on that described previously for the ewe (Leung & Wathes 1999). Strips of intercotyledonary endometrium were dissected, transferred to defined basic medium and chopped into 1 mm^3 using a mechanical tissue chopper (McIlwain Laboratory Engineering Ltd, Guildford, Surrey, UK). The endometrial pieces were blotted with a sterile lens cleaning tissue and weighed to provide 0·15 g per culture dish. They were placed on a metal grid (stainless steel, 30 mm × 30 mm × 0·5 mm) cushioned with a layer of lens cleaning tissue in dishes (50 mm × 15 mm, single

dent; BDH, Poole, Dorset, UK) containing 6 ml treatment medium (see below). The tissues were cultured at 37 °C and 5% CO_2 for 48 h in a humidified incubator. Medium was changed at 24 h.

Medium preparation

The basic medium used was Dulbecco's MEM/nutrient F12 mix (1:1) with 15 mM Hepes (Gibco BRL Life Technologies Ltd, Paisley, Strathclyde, UK) containing penicillin sodium (50 IU/ml; NVS, Fenton, Stoke-on-Trent, Staffs, UK), streptomycin sulphate (50 mg/ml; Sigma), 1·125 g/l sodium hydrocarbonate and BSA (1·125 g/l; Sigma). The medium was filtered (0·22 μm filter; Millipore, Watford, Herts, UK). Finally, 1 ml insulin–transferrin–selenium mixture (ITS; Sigma) was added to 1 l of filtered medium to provide final concentrations of 5 μg/l, 5 μg/l and 5 ng/l respectively. The medium was stored at 4 °C until used.

Stock solutions of DEX (Sigma) and LPS from *E. coli* 026.B6 (Sigma) were prepared in absolute and 75% ethanol respectively. Human recombinant interleukins (hrIL) were from Amersham International plc, Amersham, Bucks, UK. Stock solutions of hrIL-1α and -6 were prepared in single strength PBS (1 × PBS; 0·13 M NaCl, 0·007 M Na_2HPO_4) with BSA (0·1% w/v). The stock solution of hrIL-2 was dissolved in HCl (pH 2·3) before diluting in PBS. The oIFN-τ (anti-viral activity $5·6 \times 10^7$ units/ml, University of Nebraska, Lincoln, NE, USA) was dissolved in 20 mM Tris–HCl, pH 7·5.

Experimental explant culture treatments

Each individual treatment was tested on tissues collected from at least three separate cows; the number of animals used in each experiment is given in the results section. Explant samples (in triplicate for each treatment) were collected at the end of the culture period (48 h) for measurement of OTR mRNA expression by *in situ* hybridisation. They were mounted in Cryo-M-bed (Bright, Huntingdon, Cambs, UK), frozen on dry ice and stored at −80 °C. Medium samples were collected for measurement of $PGF_{2\alpha}$ and PGE_2 produced between 24 and 48 h *in vitro* (in duplicate for each treatment) and stored at −20 °C for RIA. The viability of the explants after 48 h in culture was examined by histological staining with haematoxylin and eosin.

Three experiments were performed. Experiment 1 examined the effect of LPS and DEX. The treatments were: (i) control (basic serum free medium); (ii) LPS (0·1 μg/ml); (iii) DEX (5 μM); and (iv) LPS (0·1 μg/ml)+DEX (5 μM). Experiment 2 examined the effect of oIFN-τ. The treatments were: (i) control (basic serum free medium); or (ii) oIFN-τ (10^6 units/ml). Experiment 3 examined the effects of various interleukins. The treatments were: (i) control (basic serum free medium); (ii) hrIL-1α (0·1 ng/ml); (iii) hrIL-2 (1 ng/ml); and (iv) hrIL-6 (1 ng/ml). These doses of interleukin were chosen to exceed the ED_{50} in cell proliferation assays by approximately 10-fold, based on the manufacturer's information (Amersham International).

In situ *hybridisation*

Whole cross sections (10 μm thick) of uterine endometrium collected at the start of the experiment and of endometrial explants collected after 48 h in culture and frozen in blocks of Cryo-M-bed were cut and thaw mounted on poly-L-lysine (0·1 mg/ml) coated slides. The sections were fixed in paraformaldehyde (4% w/v) in 1 × PBS for 5 min, washed in 1 × PBS for 2 min (three times) and dehydrated in 75% followed by 95% absolute ethanol for 5 min each. The sections were then stored in 95% absolute ethanol at 4 °C until used.

The *in situ* hybridisation procedures were applied as described previously (Wathes *et al.* 1996, Leung & Wathes 1999). Briefly, the OTR DNA probe (antisense; 45 mer synthetic oligonucleotide), end labelled with [^{35}S]dATP (Amersham International) was added to the sections (10^5 c.p.m./100 μl hybridisation buffer per slide), covered with a parafilm coverslip, and then incubated at 43 °C overnight. After incubation, the sections were washed at room temperature for 30 min followed by 1 h at 55 °C in 1 × sodium saline citrate (15 mM sodium chloride, 15 mM sodium citrate, pH 7·0) containing 0·2% (w/v) sodium thiosulphate pentahydrate. The slides were then dehydrated in a gradient of ethanol, air-dried and exposed to hyperfilm-β_{max} (Amersham International) for 2 weeks. A labelled oligonucleotide of the sense sequence was used as the negative control for each experiment. Sections from a bovine uterus collected at oestrus were used as the positive control in each batch of sections processed.

Photographic emulsions

The procedures were similar to the instructions provided by the manufacturing company (LM-1; Amersham International). Completely dried slides were dipped into the emulsion vertically for 5 s at 43 °C and allowed to dry horizontally at room temperature and then on a metal plate pre-cooled with dry ice for 10 min each. The slides were then placed into a light-tight box with anhydrous silica gel, sealed, and incubated at 4 °C for 3 weeks. After incubation, the slides were dipped into developer (Phenisol; Ilford Limited, Ilford, Essex, UK) for 5 min, stop bath (0·5% acetic acid v/v) for 1 min, fixative (sodium thiosulphate pentahydrate (47% w/v)) for 10 min and then distilled water for at least 10 min before counterstaining with Harris' haematoxylin and eosin to identify the cell types.

RIA

The method used for the $PGF_{2\alpha}$ and PGE_2 assays was similar to that described previously by Higgins & Lees (1984). Briefly, the medium was diluted 100-fold with Tris–HCl buffer (40 mM, pH 7·4). The diluted samples (100 μl in duplicate) were then incubated with PGE_2 or $PGF_{2\alpha}$ specific antibodies (kindly donated by Professor N L Poyser, Department of Pharmacology, University of Edinburgh, Edinburgh, UK) overnight at 4 °C. The $PGF_{2\alpha}$ antibody cross reacted 0·54% with PGE_1, 0·17% with PGE_2 and 0·21% with 13,14 dihydro-15-keto-$PGF_{2\alpha}$ and was used at a working dilution of 1:12 000. The PGE_2 antibody cross reacted 48·7% with PGE_1, 0·47% with $PGF_{2\alpha}$ and 0·16% with 13,14 dihydro-15-keto-PGE_2 and was used at a working dilution of 1:3000. [^{3}H]$PGF_{2\alpha}$ (7·4 MBq/ml) and [^{3}H]PGE_2 (3·7 MBq/ml) were from Amersham International. Separation was achieved with charcoal/dextran and ^{3}H in the supernatant was then counted on a scintillation counter for 1 min in 4 ml of scintillant (Packard Bioscience BV, Pangbourne, Berks, UK). The intra-assay coefficients of variation for PGE_2 and $PGF_{2\alpha}$ were 3·5 and 4·1% respectively. The samples from each experiment were measured at the same time to eliminate any inter-assay variation. The detection limits for PGE_2 and $PGF_{2\alpha}$ were 0·04 and 0·01 ng/ml respectively.

Cell transfection experiments

The effects of IL-1 and -2 on OTR expression were also tested in CHO cells. Reporter constructs were prepared with a bovine OTR gene PstI fragment containing 1169 bp upstream from the transcription start site cloned into pCAT basic (Promega, Southampton, Hants, UK).To provide the OTR genomic sequence required, bovine DNA was prepared from endometrium and a -1169, $+1179$ bp fragment encompassing the bovine OTR gene upstream and first intron regions was amplified by PCR using primers based on the sequence described by Bathgate *et al.* (1995). After subcloning in Bluescript and sequencing, a PstI fragment ($-1169, +146$ bp relative to the transcription start site) was cloned in the pCAT basic vector. pCAT control was used as control vector.

Transfection conditions were optimised for voltage and capacitance as well as for DNA concentration and medium composition. Cells were electroporated at 320 V and 1200 μF with 5 μg DNA in a volume of 800 μl; medium was changed 24 h after electroporation and interleukins added. Cultures were then terminated 24 or 48 h later by addition of reporter lysis buffer and processing for chloramphenicol acetyl transferase (CAT) and protein assays (Flint *et al.* 2000).

Data analysis

Images of the films from the *in situ* hybridisation autoradiography experiments were quantified by measuring the absorbance of specific areas (identified using haematoxylin and eosin staining) using an image analysis system (Seescan plc, Cambridge, Cambs, UK) as described previously (Leung & Wathes 1999). The results were expressed as arbitrary optical density units (OD) on a linear scale from 0 to 2·1. Measurements were made of four slides per sample (two antisense and two sense), with two sections per slide. As expression of OTR was confined to LE (see Fig. 1), measurements were only taken from this cell type with three regions measured per section, giving a total of 12 initial readings per sample. The sense values were subtracted from the antisense to produce a mean value of specific hybridisation in LE for each sample. The detection limit was taken as OD>0·01. All the samples from the same tissue culture experiment were processed for *in situ* hybridisation at the same time to remove any between batch variation in the analysis of treatment effects. The inter batch coefficient of variation (calculated from the positive control sections from a cow in oestrus) was 12%.

Data obtained from RIA and *in situ* hybridisation from each treatment were analysed by two way ANOVA using an SPSS software package (SPSS Inc., Chicago, IL, USA) and a general linear model routine. This method took account of the variances of treatments and cows and their interactions. Where significance was established (defined as $P<0·05$), subsequent multiple comparisons between treatment groups were performed using an LSD test.

Results

Measurements of the OTR mRNA concentration in the uterine LE at the time of initial tissue collection revealed that the 13 cows used fell into two groups. In nine cows no OTR were detected (OD in LE<0·01). In the remaining four cows the OTR mRNA had up-regulated, giving a mean OD in LE of $0·10 \pm 0·009$ and indicating that LS had already been initiated (Robinson *et al.* 1999) (Fig. 1). These two groups were designated late luteal phase (LLP) or luteolysis (LS). The mean day of the oestrous cycle on which tissue was obtained was $15·4 \pm 0·50$ in LLP and $16·4 \pm 0·24$ in LS. As the results of the treatments obtained from these two groups differed, all subsequent analyses were performed separately for the two groups. This information was not available at the time that the cultures were set up, leading to variation in the number of animals exposed to each treatment *in vitro*.

Experiment 1: the effect of LPS and DEX on OTR expression and prostaglandin production

These experiments were performed on tissue from three cows, all of which were in the LLP group. OTR mRNA expression was therefore undetected (OD<0·01) in the whole uterine samples. Spontaneous up-regulation of

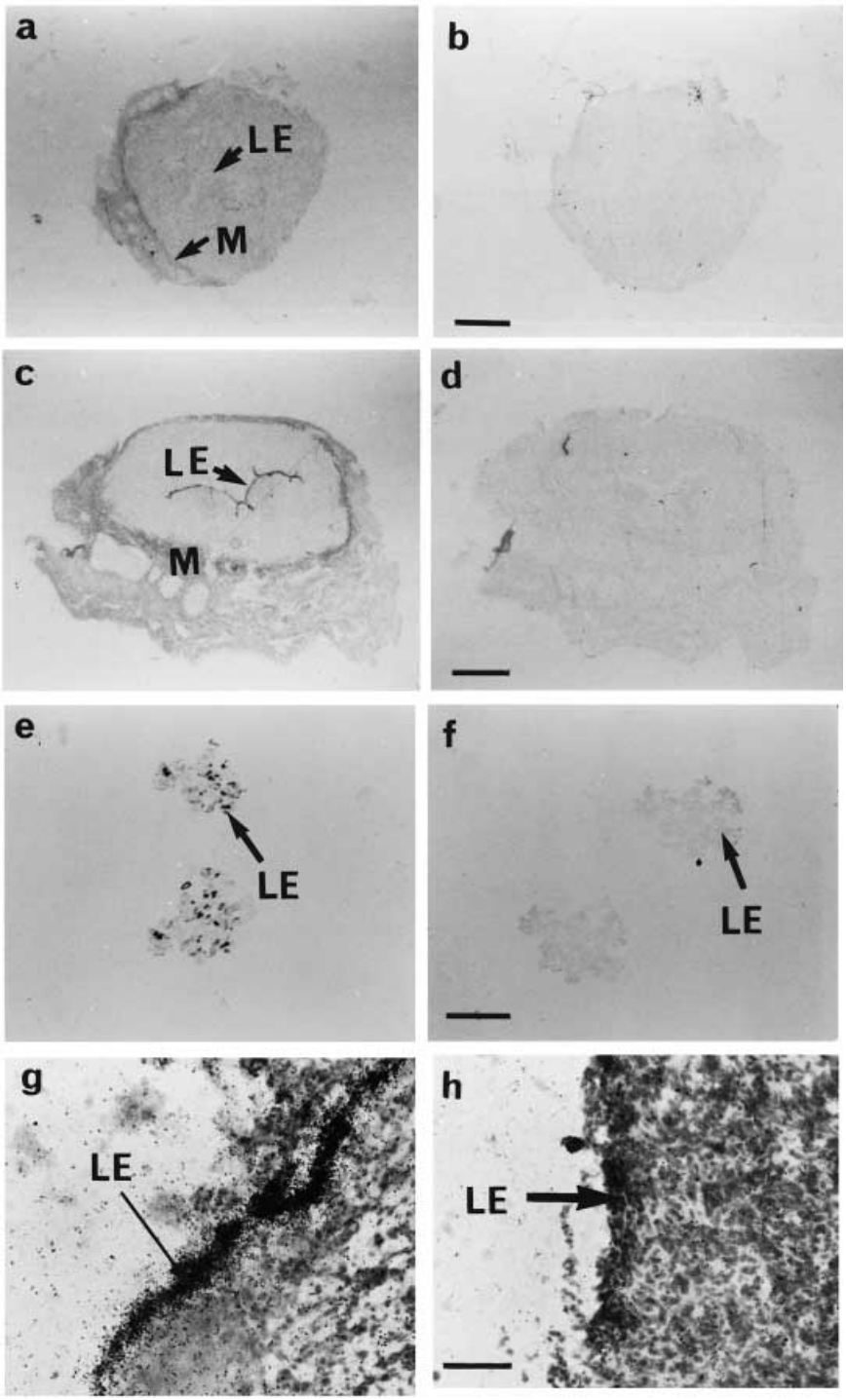

Figure 1 (a–d) OTR mRNA expression in cross sections of bovine uterus. (a, b) Uterus collected in LLP (day 17) before any up-regulation had occurred in LE, although there is some expression in myometrium (M). (c, d) Uterus collected on day 16 after LS had been initiated as determined by the clearly detectable OTR mRNA expression in LE. (e–h) OTR mRNA expression in (e, f) autoradiographs and (g, h) emulsion coated slides of endometrial explants collected after 48 h *in vitro*. (a, c, e, g) were treated with antisense probe and (b, d, f, h) with the sense (control) probe. OTR mRNA was up-regulated in culture in endometrium from cows with an initial low OTR mRNA concentration, with expression confined to LE The scale bars represent 2·5 mm in (a–d), 6 mm in (e, f) and 20 µm in (g, h).

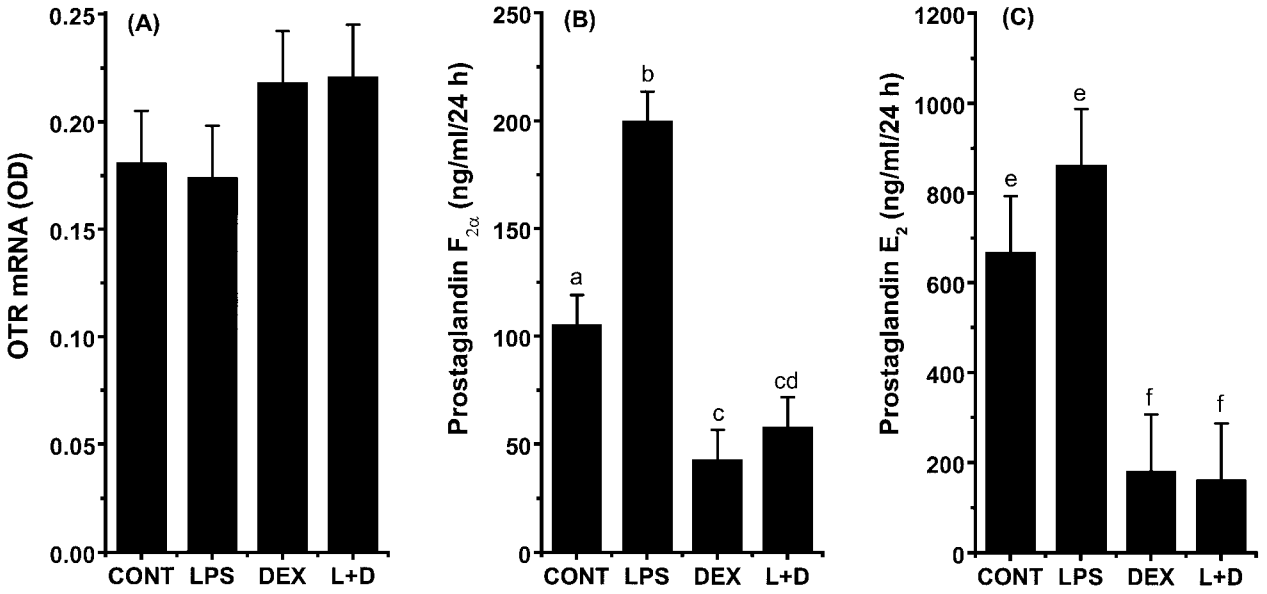

Figure 2 The effects of LPS and DEX alone or in combination (L+D) on: (A) OTR mRNA expression measured in OD units, (B) $PGF_{2\alpha}$ production and (C) PGE_2 production. Bovine endometrial explants were treated with 0·1 μg/ml LPS, 5 μM DEX or both together for 48 h. Medium was changed at 24 h and prostaglandins and OTR mRNA were measured at 48 h. Each treatment was tested on tissue from three separate cows obtained in LLP. The data are the estimated marginal means ± S.E. from the ANOVA: b>a, $P<0·001$; c<a, $P<0·007$; d<a, $P<0·03$; e>f, $P<0·02$.

OTR mRNA expression in the endometrial explants occurred within 48 h, localised exclusively to the endometrial LE (Fig. 1). The concentration of OTR mRNA at 48 h was not affected by treatment with LPS or DEX, alone or in combination (Fig. 2A). In contrast, LPS alone stimulated the production of $PGF_{2\alpha}$ ($P<0·001$), whereas DEX alone was inhibitory ($P<0·01$). With PGE_2 the pattern of effects was similar although the increase in the presence of LPS was not statistically significant. For both prostaglandins the inhibitory effect of DEX prevented LPS stimulation (Fig. 2B and C).

Experiment 2: the effect of oIFN-τ on OTR expression and prostaglandin production

The three cows used for these experiments were all in the LS group. The concentration of OTR at sample collection was 0·10 ± 0·01 OD units, and the concentration 48 h later, although slightly higher, was not significantly different (0·14 ± 0·04, $P>0·05$). IFN-τ reduced OTR mRNA concentration to 0·03 ± 0·14 OD units (Fig. 3A; $P<0·001$) but increased production of both $PGF_{2\alpha}$ and PGE_2 (Fig. 3B and C; $P<0·005$ in both cases).

Experiment 3: the effects of IL-1α, -2 and -6 on OTR expression and prostaglandin production

The interleukin experiments were performed on five cows in the LLP group and four cows in the LS group, although not every treatment was replicated in every animal. In the LLP cows, OTR was undetected at the start of culture but had increased to an OD of 0·16 ± 0·014 in the control dishes after 48 h *in vitro*. Treatment with IL-1α, -2 or -6 suppressed endometrial OTR mRNA expression (Fig. 4A). IL-1α and -2 stimulated $PGF_{2\alpha}$ production, whereas IL-6 did not (Fig. 4B). The pattern of effects of the interleukins on PGE_2 production was similar, with a tendency to increase following addition of IL-1α and -2 (overall treatment effect $P=0·17$, Fig. 4C).

In the LS group, OTR mRNA concentrations were 0·10 ± 0·009 OD units at the start of the experiment and 0·13 ± 0·015 after 48 h in control medium. This 48 h value was similar to that found in the LLP cows, but the effect of the interleukins on OTR mRNA expression was no longer apparent. Indeed the trend, although failing to reach statistical significance (overall treatment effect $P=0·11$), was in the opposite direction (Fig. 4D). The effects on prostaglandin metabolism also differed. The basal production of $PGF_{2\alpha}$ in the control dishes tended to be higher than in the LLP cows (LS 176 ± 56·7 cf. LLP 83·5 ± 17·8 ng/ml) but the difference was not significant and none of the interleukin treatments caused a significant further increase although there was again a tendency for IL-2 to cause stimulation. Basal levels of PGE_2 were not significantly different to start with (LS 475 ± 106·4 cf. LLP 299 ± 97·5 ng/ml) but were significantly increased above control levels by IL-2 only (Fig. 4F).

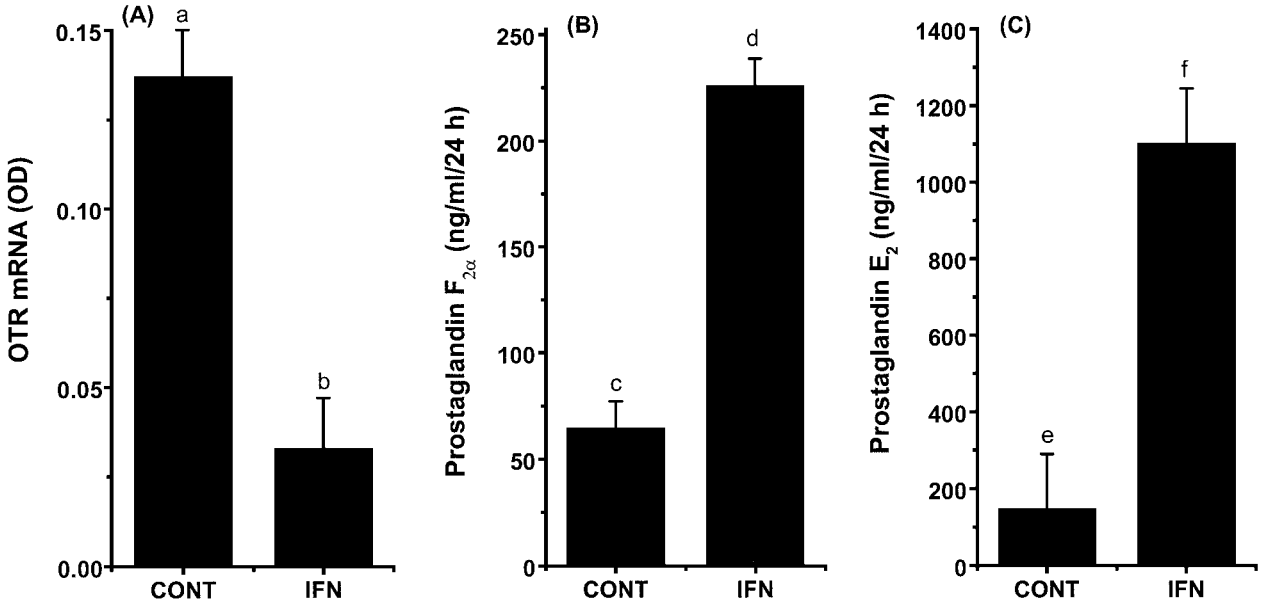

Figure 3 The effects of oIFN-τ on: (A) OTR mRNA expression measured in OD units, (B) $PGF_{2\alpha}$ production and (C) PGE_2 production. Bovine endometrial explants were treated for 48 h with 10^6 units/ml oIFN-τ. Medium was changed at 24 h and prostaglandins and OTR mRNA were measured at 48 h. Each treatment was tested on tissue from three separate cows obtained after the initial onset of LS. The data are the estimated marginal means ± S.E. from the ANOVA: a>b $P<0{\cdot}001$; d>c, $P<0{\cdot}001$; f>e, $P<0{\cdot}003$.

Cell transfection experiments

There was no effect of IL-1α (0·1 or 1 ng/ml) or IL-2 (1 or 10 ng/ml) on CAT activity in CHO cells transfected with the bovine upstream promoter of the OTR at 24 or 48 h. pCAT control induced CAT expression at approximately 50-fold the rate following transfection with pCAT-OTR, and this was also unaffected by IL-1α and -2. The CHO cells were, however, responsive to IL-1α and -2 in terms of altered MAP kinase phosphorylation (E L Sheldrick, unpublished observations).

Discussion

In this study we have investigated the possible roles of various interleukins and LPS in the luteolytic process using a bovine endometrial explant culture system. The results have confirmed previous observations that OTR expression (both mRNA and protein) in both ovine and bovine endometrium is spontaneously and significantly up-regulated *in vitro* in the absence of any exogenous hormonal treatments (Horn *et al.* 1998, Leung & Wathes 1999, 2000), but is inhibited by IFN-τ.

IL-1, -2 and -6 are all pro-inflammatory cytokines which can be expressed in endometrium. IL-1α and -1β are both glycosylated proteins of 17 kDa. Although they only share 25% sequence homology they act through the same IL-1 receptor and have similar biological activity. IL-1α mRNA is present in the endometrium of both pregnant and non-pregnant cows on day 16 after oestrus (Leung *et al.* 2000). IL-1 protein was present in bovine uterine flushes collected on days 14 and 17, but was absent on days 25 and 30, suggesting that its production may be inhibited at the time of blastocyst attachment (Davidson *et al.* 1995). IL-1 stimulates uterine prostaglandin production, in part at least by increasing the expression of the cyclo-oxygenase COX-2 (Bany & Kennedy 1995, Chen *et al.* 1995, Kniss *et al.* 1997). We have confirmed that IL-1α stimulates endometrial $PGF_{2\alpha}$ production in LLP and shown that it also inhibits endometrial OTR expression in endometrial explants collected at this time. It did not, however, have any significant effect on either prostaglandin production or endometrial OTR mRNA expression once LS had already been initiated. This could suggest a loss of IL-1 receptors at this time; alternatively IL-1 might be ineffective when both OTR mRNA and prostaglandin production are already highly stimulated *in vivo*.

IL-2, a potent pro-inflammatory cytokine, is a key component of an immune response. It induces proliferation of activated T and B cells and stimulates the cytotoxicity of T cells and natural killer cells. Raised levels of IL-2 in the placenta are associated with spontaneous abortion in mice (Tangri & Raghupathy 1993) and women (Hill *et al.* 1995, Marzi *et al.* 1996). Whereas IL-1 mRNA was present in endometrium of both pregnant and non-pregnant cows on day 16, IL-2 mRNA was only present in the non-pregnant endometrium, suggesting that its expression is down-regulated in early pregnancy (Leung

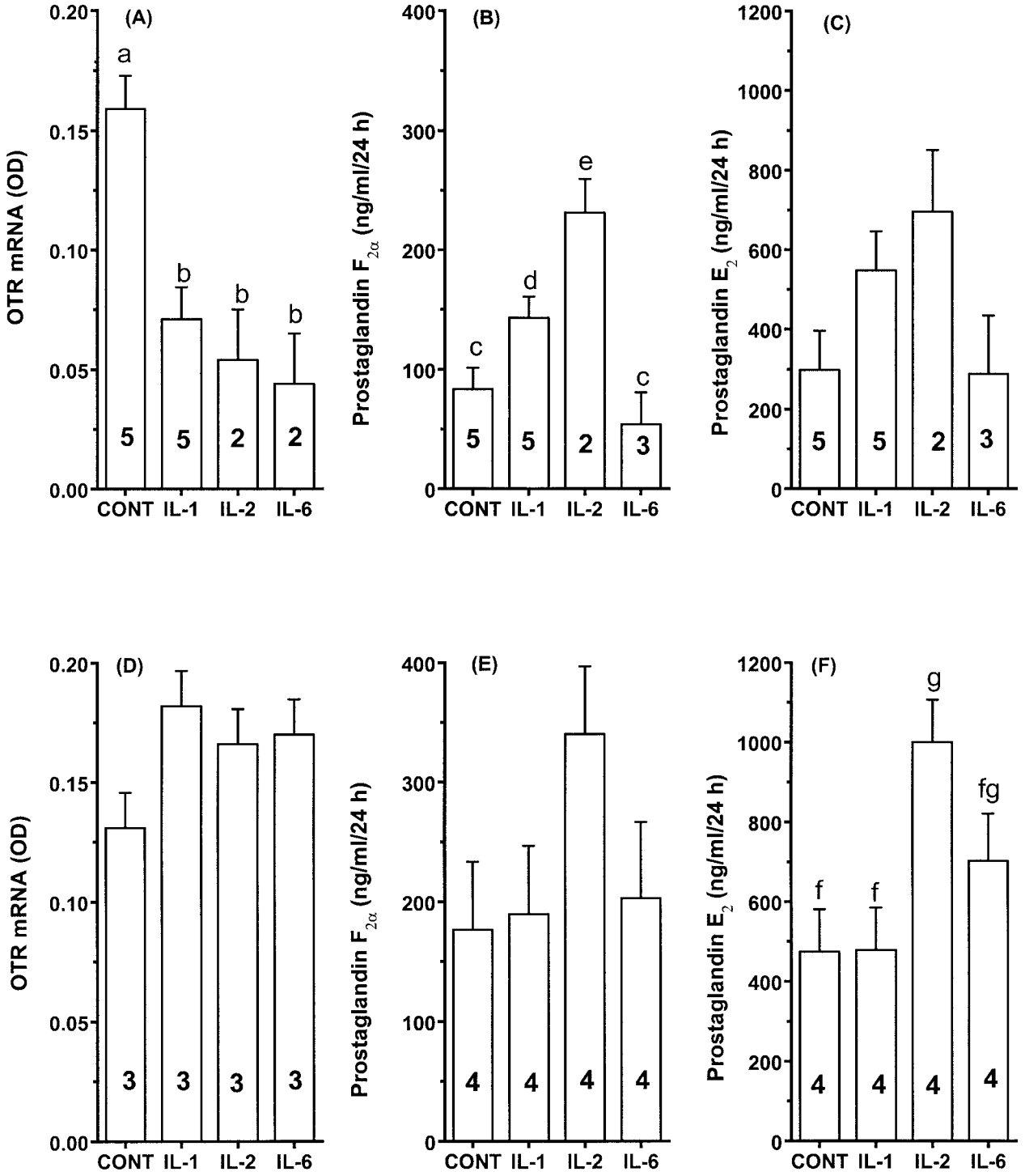

Figure 4 The effects of IL-1α, -2 and -6 on: (A, D) OTR mRNA expression measured in OD units, (B, E) $PGF_{2\alpha}$ production and (C, F) PGE_2 production. Bovine endometrial explants were treated for 48 h with 0·1 ng/ml hrIL-1α, 1 ng/ml hrIL-2 or 1 ng/ml hrIL-6. Medium was changed at 24 h and prostaglandins and OTR mRNA were measured at 48 h. Results are shown separately for endometrium collected from cows in LLP before OTR mRNA up-regulation had been initiated (A–C) and from cows in the early stages of LS, after OTR mRNA had appeared in LE *in vivo* (D–F). The number of animals receiving each treatment *in vitro* are shown on the individual bars. The data are the estimated marginal means ± S.E. from the ANOVA: b<a, $P<0{\cdot}001$; d>c, $P<0{\cdot}03$; e>c, $P<0{\cdot}001$; g>f, $P<0{\cdot}003$.

et al. 2000). PGE_2 levels are preferentially raised in early pregnancy (see below) and Emond *et al.* (1998) have shown that PGE_2 inhibits IL-2 production by bovine lymphocytes. IL-2 also stimulates prostaglandin synthesis and our studies revealed an interesting difference in the pattern of effects produced before and just after the onset of LS. In the endometrium collected from the LLP cows, IL-2 was a strong stimulator of $PGF_{2\alpha}$ and also tended to increase PGE_2. In endometrium collected on average 1 day later, but in which OTR expression had already up-regulated, the stimulatory effect of IL-2 on $PGF_{2\alpha}$ was no longer quite significant, whereas the effect on PGE_2 was more pronounced. Several studies with cultured bovine endometrial cells have shown that $PGF_{2\alpha}$ is primarily produced by the epithelial cells, whilst PGE_2 is the main product of stromal cells (Danet-Desnoyers *et al.* 1994, Kim & Fortier 1995, Asselin *et al.* 1996). The increase in PGE_2 in response to IL-2 after LS may reflect changes in the distribution of the IL-2 receptor within the endometrium at this time and/or a change in steroidal environment. Although this was not measured, we would expect that, following the increase in OTR, progesterone would start to decline while oestradiol levels would rise.

IL-6 is a glycoprotein of 22–29 kDa which is a member of the immunoglobulin superfamily. Cells of the immune system only produce IL-6 following appropriate stimulation by a variety of agents including LPS, IL-1, tumour necrosis factor and viruses (Akira *et al.* 1990, Tabibzadeh 1991). IL-6 mRNA was not detected in day 16 bovine endometrium from normal cows by PCR (Leung *et al.* 2000) but it was found in bovine conceptuses collected on days 16–20 (Mathialagan *et al.* 1992) and it has also been reported in both human (Tabibzadeh *et al.* 1989) and mouse (Jacobs *et al.* 1992) endometrium. In our studies, IL-6 had effects similar to IL-1 and -2 on OTR expression, reducing OTR expression before the onset of LS but not after. IL-6 did not, however, alter prostaglandin production.

The fact that hrIL-1α, -2 and -6 all produced responses in the bovine explant culture system confirmed that these preparations are effective on bovine tissues and that the concentrations used in this study were appropriate. All three interleukins tested showed similar effects on OTR expression, causing a marked inhibition in endometrium collected in LLP, whereas in cows in which OTR up-regulation had already occurred there was a non-significant tendency to increase OTR expression. The difference in response in explants collected before and after OTR up-regulation *in vivo* may be caused by a shift in steroidal environment between the two sets of samples as discussed above. The similarity in responses to the three interleukins suggests that these inflammatory interleukins may all stimulate an intermediary inhibitory factor which regulates OTR expression rather than having a direct action. NF-IL-6, a member of the c/EBP family, would be a potential candidate. This nuclear transcription factor binds to the regulatory region of several acute phase and cytokine regulated genes (Akira *et al.* 1990). Furthermore, NF-IL-6 mRNA is induced by many cytokines including IL-1 and -6 (Akira *et al.* 1990). It was not, however, possible to demonstrate an effect of IL-1 or -2 in CHO cells transfected with the bovine OTR promoter, although the cells were responsive to IL-1α and -2 as indicated by MAP kinase phosphorylation (E L Sheldrick, unpublished observations). This may suggest that the required transcription factors were not present or could not be induced in these cells. Of the six NF-IL-6 sites present in the rat and human OTR promoter, only two are also represented in the bovine OTR promoter region. Both these sites are included in the bovine OTR promoter sequence used here at 889 and 1054 bp from the 5′ end. Acute phase response factor (APRF) and NF-1 sites are also present at 497, 646 (NF-1) and 903 bp (APRF). Attempts to test the effects of interleukins on OTR promoter function in Madin–Darby bovine kidney cells and in a passageable bovine stromal cell line were unsuccessful due to the low expression of the promoter construct in these cells.

The effects of IFN-τ in the endometrial explant system agree with previous studies. IFN-τ alone, in the absence of progesterone, down-regulated OTR expression (Asselin *et al.* 1997*a*, Xiao *et al.* 1999). This effect was particularly notable as all the cows tested had already started to increase OTR expression *in vivo*. At the same time, basal prostaglandin production of both $PGF_{2\alpha}$ and PGE_2 was stimulated presumably reflecting the up-regulation of phospholipase A_2 (Hannigan & Williams 1991) and COX-2 expression (Asselin *et al.* 1997*b*). IFN-τ also shifted the predominant prostaglandin produced by the epithelial cells from $PGF_{2\alpha}$ to PGE_2. The net result would be a preferential increase in PGE_2 levels following IFN-τ stimulation by the embryo in early pregnancy. In contrast, Danet-Desnoyers *et al.* (1994) found that recombinant bovine IFN-τ inhibited both $PGF_{2\alpha}$ and PGE_2 in bovine epithelial cells but had no effect on stromal cells. The reason for this discrepancy is unclear.

An acute immune response induced by bacterial endotoxins such as LPS leads to increased production of prostaglandins in many cell types including macrophages, fibroblasts, lymphocytes and epithelial cells. Cytokines such as IL-1α and -2 represent one of the mechanisms responsible (Dinarello 1996, Hirsch *et al.* 1999). Prolonged secretion of $PGF_{2\alpha}$ has been reported in *post partum* cows in association with bacterial uterine infection (Bekana *et al.* 1996). In the human uterus there is evidence that LPS may promote prostaglandin synthesis by inducing the release of phospholipase A_2 (Farrugia *et al.* 1999). In this study, we demonstrated that LPS increased $PGF_{2\alpha}$ production in the bovine endometrium without altering the secretion of PGE_2 significantly. We have also confirmed that DEX, a glucocorticoid analogue, suppressed the production of $PGF_{2\alpha}$ stimulated by LPS, as previously demonstrated in other cell systems (Dinarello 1996,

Bleeker *et al.* 1997). In addition, DEX alone inhibited the basal production of both prostaglandins. Glucocorticoids inhibit COX expression in inflammatory cells (Masferrer *et al.* 1992, O'Banion *et al.* 1992), although the opposite action can occur in some situations; for example cortisol increases PGE_2 synthesis by amnion in late pregnancy (Zakar *et al.* 1995). Betamethasone treatment increased the length of the luteal phase in heifers (Kanchev *et al.* 1976). These results raise the possibility that stress could modulate uterine prostaglandin synthesis in the cow via raised corticosteroid production. Whilst LPS and DEX had clear effects on prostaglandin synthesis, they did not alter endometrial OTR expression.

In summary, in the non-pregnant cow during the luteal phase the pro-inflammatory interleukins may form part of the inhibitory mechanism with progesterone to help maintain OTR suppression, while at the same time enhancing basal prostaglandin synthesis. This increase in prostaglandin would not activate LS in the absence of OTR, since pulsatile secretion of $PGF_{2\alpha}$ is required (McCracken *et al.* 1999). Our data do not, therefore, suggest that up-regulation of endometrial OTR expression is controlled by an acute inflammatory reaction. Prostaglandin synthesis can, however, be stimulated as part of a defence mechanism to inflammatory diseases such as endometritis without necessarily inducing LS. Once LS has been initiated, by a mechanism which remains unclear, the effects of interleukins on OTR expression are lost. The similarity of effect of IL-1α, -2 and -6 on OTR expression suggests that they may all act through the same intermediary mechanism although they each have distinct effects on $PGF_{2\alpha}$ and PGE_2 synthesis. In early pregnancy the embryo produces IFN-τ, which both inhibits OTR development and enhances endometrial PGE_2 production. The raised levels of PGE_2 may then be responsible for the decrease in IL-2 mRNA at this time, which we have reported previously (Leung *et al.* 2000).

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