Correlation of increased concentration of ovine endometrial cyclooxygenase 2 with the increase in PGE₂ and PGD₂ in the late luteal phase

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Ovine endometrium showed transient expression of high concentrations of the inducible isoform of cyclooxygenase, cyclooxygenase 2 (COX-2), whereas the constitutive isoform, cyclooxygenase 1 (COX-1), was expressed at much lower concentrations and did not change. In this study, the pattern of prostaglandin synthesis in endometrial luminal cells was investigated in relation to their COX-2 content. Endometrial cells from cyclic or pregnant ewes at days 9, 12, 14 and 16 were isolated and analysed for the presence of COX-1 and COX-2 proteins using western blot analysis. Freshly isolated cells were incubated with 0.5 µCi [³H]arachidonic acid ml⁻¹. Radioactive cyclooxygenase metabolites were analysed by reverse-phase HPLC. Luminal cells produced mainly PGF_{2α}, PGE₂, PGD₂ and 13,14-dihydro-15-keto PGF_{2α} and to a lesser extent 6-keto PGF_{1α}, thromboxane B₂ and 13,14-dihydro-15-keto PGE₂. The production of PGE₂ and PGD₂ was proportional to the cellular concentration of COX-2. PGE₂ and PGD₂ release was low on day 9 when COX-2 was not expressed, whereas high concentrations of PGE₂ and PGD₂ were synthesized on days 12–14 when COX-2 was highly expressed, reaching 100 ng µg⁻¹ cellular protein. In contrast, the basal production of PGF_{2α} did not appear to be related to COX-2 concentration and was greatest on day 16. Moreover, the release of PGF_{2α} was maintained at steady state values between days 9 and 14 by the production of 13,14-dihydro-15-keto PGF_{2α}. Although PGF_{2α} output was lower at day 16 of pregnancy compared with the oestrous cycle, no difference was observed in the pattern of prostaglandin synthesis between pregnant and non-pregnant ewes.

Introduction

The endometrium is a major site of prostaglandin synthesis in ruminant species as in other mammals (Poyser, 1995). Prostaglandin production depends on the activity of cyclooxygenase enzymes responsible for the conversion of arachidonic acid to the intermediate precursor PGH₂. PGH₂ is metabolized into one or more of the primary biologically active prostaglandins according to the enzymatic capacity of the cells. There are two isoforms of cyclooxygenase: COX-1 and COX-2. COX-1 is constitutively expressed in many types of cell and is thought to perform housekeeping functions. In contrast, COX-2 is normally absent from cells and is induced as an early responsive gene by a wide variety of factors (Herschman, 1994; Goppelt-Strube, 1995). COX-2 is expressed in epithelial cells from ovine endometrium and increases markedly during the late luteal phase of the oestrous cycle, whereas COX-1 is expressed at low steady state concentrations (Charpigny *et al.*, 1997a). These findings indicate that COX-2 from epithelial cells is responsible for the

luteolytic surge of PGF_{2α} synthesis observed at the end of the oestrous cycle. This hypothesis is consistent with reports showing that epithelial cells are the primary source of PGF_{2α} in the endometrium (Fortier *et al.*, 1988; Cherny and Findlay, 1990; Danet-Desnoyers *et al.*, 1994; Asselin *et al.*, 1996). However, the persistence of high concentrations of COX-2 in early pregnancy (Charpigny *et al.*, 1997a) indicates that COX-2 expression is not only relevant to luteolysis, but that COX-2-dependent prostaglandins have a role in the establishment of pregnancy. The finding of Asselin *et al.* (1997a,c) that conceptus IFN-τ triggers an important increase of PGE₂ production by bovine endometrial cells via upregulation of COX-2 gene expression supports this hypothesis. However, the physiological significance of these *in vitro* experiments remains unclear. In spite of efforts to improve endometrial cell culture systems, such as maintenance of cellular polarity (Cherny and Findlay, 1990; Classen-Linke *et al.*, 1997), it is likely that endometrial cell monolayers do not maintain their normal cellular function in culture. The expression of COX-2 is tightly regulated by steroid hormones (Charpigny *et al.*, 1997a; Wu *et al.*, 1997). The presence of steroids in culture medium may result in a disruption of COX-2 control

Received 22 January 1999.

mechanisms. Moreover, fetal bovine serum, which is generally required for endometrial cell culture, is a strong inducer of COX-2 in many cell systems (O'Banion *et al.*, 1991; Xie and Herschman, 1996). This type of culture-related artifact may give a distorted picture of the qualitative and quantitative contributions made by the cells to endometrial prostaglandins.

The purpose of this study was to evaluate the contributions of the different types of endometrial cell to prostaglandin synthesis using freshly prepared ovine cells to avoid functional changes due to the culture. The aim was to establish the pattern of prostaglandin synthesis in relation to endometrial cyclooxygenase content from ewes at different days of the oestrous cycle and of early pregnancy.

Materials and Methods

Animals and isolation of endometrial cells

All procedures relating to the care and use of animals were approved by the French Ministry of Agriculture according to the French regulations (instruction 19/04/1988) for animal experimentation.

Préalpes-du-Sud ewes were used in all experiments. Oestrus was synchronized using intravaginal sponges containing 60 mg 6 α -methyl-17 α -acetoxypregesterone for 14 days as described by Charpigny *et al.* (1997a). On the day of sponge withdrawal, ewes received one i.m. injection of 500 iu pregnant mares' serum gonadotrophin (PMSG). Oestrus was observed 48 h later (day 0 of the cycle) and those animals assigned to be in the pregnant group were mated. On days 9, 12, 14 and 16 of the oestrous cycle or pregnancy, the ewes were killed and the reproductive tract was excised. Five ewes were used for each stage of the oestrous cycle or pregnancy. Conceptuses were recovered by flushing the uterine horns to confirm pregnancy. Luminal epithelium was exposed for 5 min to modified Ca²⁺- and Mg²⁺-free Hank's balanced salt solution (Sigma, St-Quentin-Fallavier). Luminal cells were separated from the underlying tissue as sheets of cells by gentle scraping with a scalpel blade. Cells were dispersed in a solution of Earle's medium containing 15 mmol Hepes l⁻¹ and centrifuged at 500 g for 5 min. The supernatant was discarded and the pelleted cells were resuspended in the same medium. The endometrium was separated from the myometrial layer by dissection. Glandular epithelium and stromal cells were obtained as described by Danet-Desnoyers *et al.* (1994) and Tamby *et al.* (1996). Briefly, the endometrium was minced with two scalpel blades and the resulting paste was placed in five volumes of Earle's medium containing 15 mmol Hepes l⁻¹, 150 iu collagenase, 150 iu hyaluronidase and 2% (w/v) BSA. Enzymatic digestion was performed at 37°C for 90 min and stopped by centrifugation at 500 g for 15 min. The pellet was resuspended in 50 ml Earle's medium containing 15 mmol Hepes l⁻¹. Dissociation of the tissue was improved by repeatedly drawing the tissue digest into a cannula. The suspension was filtered through gauze to remove non-digested tissue. A second filtration of the suspension through a 30 μ m nylon mesh (Scrynel, PolyLabo, Paris) retained

epithelial glands as aggregates. The aggregates were recovered on the top of the nylon mesh by rinsing with Earle's buffered solution. Single cells which passed through the mesh were mostly stromal cells. Single cell suspensions and cell aggregates were centrifuged at 500 g for 5 min and the resulting pellets were resuspended in culture medium.

Incubation of cells

Cells were transferred into plastic culture dishes containing 6 ml Earle's medium supplemented with the essential and non-essential amino acids of minimum essential medium (Sigma) and containing 15 mmol Hepes l⁻¹ and allowed to sediment for 2 h at 38°C in a humidified atmosphere of 5% CO₂ in air. The incubation was initiated by the addition of 0.5 μ Ci [5,6,8,9,11,12,14,15(N)-³H]arachidonic acid ml⁻¹ (specific activity: 200 Ci mmol⁻¹; Amersham, les Ulis) and carried out for 6 h. This incubation time was sufficient for incorporation of arachidonic acid into cell lipids (Tamby *et al.*, 1996). Control culture medium containing radioactive arachidonic acid without cells was incubated under identical conditions to assess the possible instability of arachidonic acid during the incubation period. Incubations were stopped by centrifugation at 10 000 g for 15 min at 4°C and supernatants were acidified to pH 3.5 with 100 μ l ml⁻¹ of 0.5 mol citric acid l⁻¹ and stored at -80°C until HPLC analysis. Cell pellets were saved for determination of protein concentration according to Bradford (1976).

HPLC analysis of cyclooxygenase metabolites of arachidonic acid

Samples were simultaneously extracted and analysed in a single-step HPLC procedure as described by Charpigny *et al.* (1997b) with some modifications. Arachidonic acid metabolites were extracted from the culture media using a precolumn containing octadecylsilyl silica (Nucleosil C18, 30 mm $\times$ 4.6 mm; Touzart et Matignon, Les Ulis) connected to a Zorbax C18 analytical column (250 mm $\times$ 4.6 mm; Shandon, Cergy-Pontoise) via an automated switching valve. The HPLC system was from Waters Associates (St-Quentin, France) and consisted of three model 510 pumps coupled to a model 680 gradient controller, a U6K injector and an automatic six-ports switching valve. Two pumps were used to deliver the mobile phases to the separation column and the third pump was used for sample loading and extraction onto the pre-column. Products were detected using a UV detector (Water Associates, model 490) and a radioactivity detector (model Flo-One A500; Packard, Rungis) equipped with a 0.5 ml cell for liquid scintillation counting. Liquid scintillator was Flo-Scint III (Packard) and was delivered at 3 ml min⁻¹.

Samples were adjusted to 15% methanol and loaded in a 10 ml loop connected to the pre-column which had been equilibrated with 15% methanol containing 0.05% acetic acid. The pre-column was washed for 8 min at a flow rate of 2 ml min⁻¹ and was coupled to the separation column. Reverse-phase HPLC separation of cyclooxygenase

derivatives was conducted at 1 ml min⁻¹ for 32 min using isocratic elution with acetonitrile:water (36:64) containing 0.05% acetic acid. The concentration of acetonitrile in the mobile phase was raised in 20 min to 95% to clean the column. After completion of the chromatography, the pre-column was disconnected from the column flow line and washed with methanol:water:acetic acid (85.0:15.0:0.05) before it was equilibrated with the starting solvent. The column was conditioned for the next run with the isocratic mobile phase.

Standards were obtained from Cayman Chemical (Spi-Bio, Massy). PGD₂, PGE₂, PGF_{2α}, thromboxane B₂, 6-keto PGF_{1α}, 13,14-dihydro-15-keto PGF_{2α} and 13,14-dihydro-15-keto PGE₂ were obtained in individual vials and kept at -20°C under nitrogen.

Thin layer chromatography analysis of prostaglandin derivatives

Radioactive peaks that eluted in HPLC at a retention time corresponding to that of unlabelled standard were collected to confirm their identity in thin layer chromatography. Samples were dried with a stream of N₂ gas. Samples were resuspended in 10 µl chloroform:methanol (2:1) and spotted on plastic thin layer plates coated with silica gel (Merck, Nogent-sur-Marne). Chromatography plates were developed as described by Salmon and Flower (1982) in a solvent system of ethyl acetate:2,2,4-trimethylpentane:acetic acid (110:50:20) saturated with water. The dried plates were placed in iodine vapour to stain prostaglandin standards. Each lane was cut into 40 pieces that were counted for radioactivity and *R_f* values of radioactive spots were compared with *R_f* values of standards. *R_f* values for standards were 0.10, 0.16, 0.22, 0.26, 0.38, 0.47 and 0.51 for 6-keto PGF_{1α}, PGF_{2α}, thromboxane B₂, PGE₂, 13,14-dihydro-15-keto PGF_{2α}, PGD₂ and 13,14-dihydro-15-keto PGE₂, respectively.

Western blot analysis

For the analysis of cyclooxygenases, freshly prepared endometrial cells were sonicated in 20 mmol Tris-HCl l⁻¹, pH 7.5, containing 5 mmol EDTA l⁻¹, 16 mmol 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate (CHAPS) l⁻¹, 1 µg leupeptin ml⁻¹, 10 µg soybean trypsin inhibitor ml⁻¹ and 1 mmol benzamidine-HCl l⁻¹. The homogenates were clarified by centrifugation at 3000 g for 15 min at 4°C. Proteins from the homogenates were separated by SDS-PAGE on a 10% gel and electroblotted onto 0.1 µm nitrocellulose membranes as described by Charpigny *et al.* (1997a,b). A detailed description of the use and preparation of anti-COX-1 L855 and anti-COX-2 mAb294 antibodies were reported by Charpigny *et al.* (1997a,b) and Créminon *et al.* (1995). Membranes to be probed with the anti-COX-2 antibody were saturated overnight at 4°C in 50 mmol Tris-HCl l⁻¹, pH 7.5, 250 mmol NaCl l⁻¹, 0.1% (v/v) Tween 20 (TBS-T buffer) containing 5% (w/v) fat-free dry milk. Membranes were then incubated for 1 h with the anti-COX-2

mAb294 at a concentration of 3 µg ml⁻¹ in TBS-T buffer containing 5% (w/v) fat-free dry milk. Membranes to be probed with the anti-COX-1 antibody were saturated overnight at 4°C with TBS-T buffer containing 1% (v/v) Tween 20. Membranes were incubated for 1 h with the anti-COX-1 L855 at dilution of 1:3000 in TBS-T buffer containing 1% (v/v) Tween 20. After extensive washes in TBS-T buffer, blots were incubated for 1 h at room temperature with goat horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (Jackson products; Interchim, Montluçon) at dilutions of 1:60 000 and 1:8000, respectively, in TBS-T buffer. The excess of the second antibody was eliminated by washing in TBS-T buffer (five consecutive 10 min washes). Chemiluminescent substrate was used according to the manufacturer's instructions (ECL Kit; Amersham) and immunoreactive proteins were visualized after 0.5–10 min exposure to hyperfilm-ECL (Amersham). Immunoreaction signals were quantified on the films by scanning densitometry using an image analysis system (M-Lecphor, Biocom). The concentration of cyclooxygenase was determined by comparison with signals of serial dilutions of sheep COX-1 and COX-2 standard proteins (Cayman Chemical).

Cells for western blot analysis of the intermediate filaments cytokeratin and vimentin were sonicated in 100 mmol phosphate buffer l⁻¹, pH 7.4, containing 16 mmol CHAPS l⁻¹, 20 mmol EDTA l⁻¹, 1 mmol dithiothreitol (DTT) l⁻¹ and 1 mmol phenylmethylsulfonyl fluoride (PMSF) l⁻¹. The homogenate was centrifuged at 10 000 g for 15 min at 4°C. The supernatant was mixed (v/v) with 100 mmol phosphate buffer l⁻¹, pH 7.0, containing 9 mol urea l⁻¹, 200 mmol DTT l⁻¹ and 4% (w/v) SDS, and heated at 50°C for 1 h. After centrifugation at 10 000 g for 15 min, proteins from the supernatant were separated by SDS-PAGE on a 12% gel and electroblotted onto polyvinylidene difluoride membrane (Hybond-P; Amersham) for 2 h. Membranes were saturated overnight in TBS-T buffer containing 5% (w/v) fat-free dry milk. Membranes were probed with anti-cytokeratin-18 mAb (clone MAD7; Eurodiagnostica, Gentilly) at a dilution of 1:3000 and with anti-vimentin mAb (clone V9; Organon Teknica, Fresnes) at a dilution of 1:2000. Incubations were carried out for 2 h at room temperature in TBST-T buffer containing 5% (w/v) fat-free dry milk. The membranes were processed as for the detection of cyclooxygenases using goat horseradish peroxidase-conjugated antimouse IgG and immunoreactive bands were visualized by enhanced chemiluminescence.

Statistical analysis

The effect of day and reproductive status on cyclooxygenase concentration and on the amount of prostaglandins released in the culture medium was tested by ANOVA using non-transformed values using the general linear model procedure from the Statistical Analysis System (SAS, Institute Inc.). Differences between individual mean concentrations of cyclooxygenase were pair-wise compared by Tukey's test. Related to the pattern of expression of cyclooxygenases, the following orthogonal contrasts were performed to test differences in prostaglandin synthesis: (1)

Fig. 1. Western blot analysis of purified luminal, glandular and stromal endometrial cells from a day 12 non-pregnant ewe. Increasing amounts of proteins from freshly prepared cells were separated by SDS-PAGE and electroblotted onto nitrocellulose or polyvinylidene difluoride (PVDF) membranes. Blots were then probed with anti-cyclooxygenase 1 (anti-COX-1) (L 855), anti-COX-2 (mAb294), anti-cytokeratin-18 (Ck18; clone MAD7) and anti-vimentin (Vim; clone V9) antibodies.

day 9 versus day 16, (2) day 12 versus day 14, (3) day 9 and day 16 versus day 12 and day 14. In addition, the production of $\text{PGF}_{2\alpha}$ and 13,14-dihydro-15-keto $\text{PGF}_{2\alpha}$ on day 16 was compared with the combined production on days 9, 12 and 14. The effect of reproductive status was examined further by day. Correlations of prostaglandin concentrations with cyclooxygenase concentrations were analysed by the Pearson product moment correlation test. Results are expressed as least square mean $\pm$ SE. The amount of radioactive arachidonic acid metabolites produced by cells was determined by integration of the radioactive peak area and was expressed in c.p.m. per 100 μg cellular proteins for a 6 h incubation period.

Results

Evaluation of cell homogeneity

Intermediate filaments vimentin and cytokeratin were detected by western blot analysis to assess the purity of the epithelial and stromal cell preparations. The different locations of COX-1 and COX-2 in the endometrium was useful in determining the purity of the cells; Charpigny *et al.* (1997a) reported that COX-2 is confined to the luminal epithelium whereas COX-1 is detected in all endometrial cells. Representative western blots for COX-1, COX-2, vimentin and cytokeratin in the different types of endometrial cell obtained from one ewe at day 12 of the oestrous cycle are shown (Fig. 1). Cytokeratin was detected in luminal and glandular epithelial cells but not in stromal

Fig. 2. Determination of (■) cyclooxygenase 1 (COX-1) and (□) COX-2 in luminal cells from ewes on days 9, 12, 14 and 16 of (a) the oestrous cycle and (b) early pregnancy. COX-1 and COX-2 proteins were quantified by scanning densitometry of enhanced chemiluminescence western blot signals relative to the chemiluminescence intensity of standard COX-1 and COX-2 proteins. Bars represent least square mean values $\pm$ SE.

cells. However, a weak signal was detected in stromal cells due to some contaminating epithelial cells. Vimentin was detected in stromal cells but not in luminal epithelial cells. A weak contamination of glandular epithelial cells by some residual fibroblasts that were not eliminated by collagenase digestion was also observed. On the basis of COX-2 content, which was highly expressed in the luminal cell preparation and was undetectable in the glandular and stromal cell preparations, contamination of glandular and stromal cells by luminal cells was estimated to be $< 1\%$. The detection of cytokeratin in stromal cells was probably due to glandular cells, but was $< 2\%$. On the basis of the detection of vimentin, contamination of luminal cells by stromal cells was $< 0.5\%$.

Determination of COX-1 and COX-2 concentration in luminal cells

The concentration of COX-1 and COX-2 in luminal cells from ewes at days 9, 12, 14 and 16 of the oestrous cycle and of pregnancy is shown (Fig. 2). COX-1 (least square mean $\pm$ SE $\text{ng } \mu\text{g}^{-1}$ cellular protein) was expressed at similar concentrations on day 9 (0.184 ± 0.03), day 12 (0.297 ± 0.026), day 14 (0.253 ± 0.027) and day 16 (0.241 ± 0.026) ($P = 0.112$). There was no difference in the concentration of COX-1 expressed in cells from pregnant (0.256 ± 0.019) and non-pregnant ewes (0.230 ± 0.020) ($P = 0.372$).

There was no difference in COX-2 concentration between pregnant and non-pregnant ewes ($P = 0.710$) and no

interaction between reproductive status and days ($P = 0.110$). However, there was a difference in COX-2 content among the different days ($P < 0.001$). COX-2 was undetectable in luminal cells on day 9, whereas COX-2 was highly expressed on days 12, 14 and 16. Luminal cells from day 16 ewes had lower concentrations of COX-2 than those from days 12 and 14 (least square mean $\pm$ SE ng μg^{-1} cellular protein: day 12 = 103.4 ± 9.0 ; day 14 = 94.6 ± 9.3 ; day 16 = 56.8 ± 8.9) (day 12 versus day 14, $P = 0.904$; day 12 versus day 16, $P = 0.004$; day 14 versus day 16, $P = 0.029$).

These data show that COX-2 expression in luminal cells is markedly upregulated during the oestrous cycle since the COX-2 protein can represent up to 10% of the total cellular protein and the mean concentration of COX-2 was 350 times greater than that of COX-1 on days 12–14.

Arachidonic acid metabolism by cyclooxygenases

The three different types of endometrial cell secreted detectable amounts of radioactive cyclooxygenase derivatives of [^3H]arachidonic acid. During a 6 h incubation period, luminal, glandular and stromal cells from day 12 non-pregnant ewes produced 136.5 ± 10.1 , 20.3 ± 2.6 and 4.8 ± 0.8 (mean $\pm$ SEM in 10^3 c.p.m. per 100 μg cellular protein) of total cyclooxygenase metabolites of [^3H]arachidonic acid in the culture medium, respectively. Luminal cells were the major source of radiolabelled arachidonic acid metabolites since these cells secreted 6.7 times more metabolites than glandular cells ($P < 0.005$) and 28 times more than stromal cells ($P < 0.001$). These results are consistent with the relative amounts of COX-1 and COX-2 proteins detected in the different types of endometrial cell (see Fig. 1).

HPLC analysis of cyclooxygenase metabolites of arachidonic acid produced by luminal epithelial cells

Representative HPLC radiochromatograms of arachidonic acid metabolites produced by luminal cells from ewes on days 9, 12 and 16 of the oestrous cycle are shown (Fig. 3). The pattern of secretion for cells from ewes at days 12 and 14 was similar. Radioactive cyclooxygenase metabolites were identified using non-radioactive standards (Fig. 3a). Moreover, peaks of radioactivity were collected and the identity of all the products was confirmed by thin layer chromatography on the basis of their R_f value. Overall, the major arachidonic acid metabolites produced by luminal cells were $\text{PGF}_{2\alpha}$, PGE_2 , PGD_2 and 13,14-dihydro-15-keto $\text{PGF}_{2\alpha}$. In addition, luminal cells secreted prostacyclin and thromboxane A2 identified by their stable metabolites 6-keto $\text{PGF}_{1\alpha}$ and thromboxane B2, respectively.

Prostaglandin production by epithelial luminal cells from ewes at days 9, 12, 14 and 16 of the oestrous cycle

Important qualitative and quantitative differences in the pattern of prostaglandin secretion from luminal cells were observed during the oestrous cycle (Fig. 4). 6-keto $\text{PGF}_{1\alpha}$ and

Fig. 3. HPLC chromatograms of arachidonic acid metabolites synthesized by luminal endometrial cells from day 9 (b), day 12 (c) and day 16 (d) non-pregnant ewes. Non-radioactive authentic standards (a) were detected by UV absorbance at 205 nm. [^3H]arachidonic acid metabolites were detected on-line with a Flo-One β counter and were identified by reference to non-radioactive standards. Their identity was confirmed by thin layer chromatography. TxB2: thromboxane B2; 6k- $\text{PGF}_{1\alpha}$: 6-keto $\text{PGF}_{1\alpha}$; dhk- $\text{PGF}_{2\alpha}$: 13,14-dihydro-15-keto $\text{PGF}_{2\alpha}$; dhk- PGE_2 : 13,14-dihydro-15-keto PGE_2 .

thromboxane B2 did not exceed 4–7% of the total radioactive cyclooxygenase metabolites throughout the oestrous cycle and there was no difference in the mean values of secreted metabolites among days (6-keto $\text{PGF}_{1\alpha}$, $P = 0.088$; thromboxane B2, $P = 0.190$). The ANOVA did not demonstrate a difference in production of $\text{PGF}_{2\alpha}$ by luminal cells among days ($P = 0.099$). However, the orthogonal contrast that compared the difference of day 16 versus days 9, 12 and 14 indicated that cells from ewes on day 16 synthesized greater amounts of $\text{PGF}_{2\alpha}$ (11000 ± 1681 c.p.m.) than those from days 9, 12 and 14 (5434 ± 2058 , 4286 ± 2058 and 4413 ± 1841 c.p.m., respectively) ($P = 0.017$). Differences among days were

Fig. 4. Cyclooxygenase metabolism of [^3H]arachidonic acid by luminal cells from ewes at (□) day 9, (▨) day 12, (■) day 14 and (▤) day 16 of the oestrous cycle. Freshly prepared cells were incubated for 6 h with 0.5 μCi [^3H]arachidonic acid ml^{-1} . Cyclooxygenase derivatives were separated by HPLC and identified. Quantification of individual cyclooxygenase derivatives was determined by integrating individual peaks of radioactivity. Histograms represent least square mean values $\pm$ SE ($\text{PGF}_{2\alpha}$: day 16 versus days 9, 12 and 14, $P = 0.017$; PGE_2 : day 9 versus day 16, $P = 0.541$; day 12 versus day 14, $P = 0.407$; days 9 and 16 versus days 12 and 14, $P < 0.001$; PGD_2 : day 9 versus day 16, $P = 0.439$; day 12 versus day 14, $P = 0.028$; days 9 and 16 versus days 12 and 14, $P < 0.001$; 13,14-dihydro-15-keto $\text{PGF}_{2\alpha}$ (dhk- $\text{PGF}_{2\alpha}$): day 9 versus day 16, $P = 0.099$; day 12 versus day 14, $P = 0.829$; days 9 and 16 versus days 12 and 14, $P < 0.001$; day 16 versus days 9, 12 and 14, $P < 0.001$; 13,14-dihydro-15-keto PGE_2 (dhk- PGE_2): day 9 versus day 16, $P = 0.005$; day 12 versus day 14, $P = 0.496$; days 9 and 16 versus days 12 and 14, $P = 0.037$).

observed for PGE_2 ($P < 0.001$), PGD_2 ($P < 0.001$), 13,14-dihydro-15-keto $\text{PGF}_{2\alpha}$ ($P < 0.001$) and 13,14-dihydro-15-keto PGE_2 ($P = 0.004$). PGE_2 production was low on day 9 (1149 ± 4138 c.p.m.) and increased 16-fold on day 12 (19020 ± 2926 c.p.m.). The synthesis of PGE_2 on day 14 remained high (16990 ± 3701 c.p.m.) and then decreased on day 16 (4780 ± 3378 c.p.m.). Orthogonal contrasts indicated that synthesis of PGE_2 on day 9 did not differ from day 16 ($P = 0.541$) and that day 12 did not differ from day 14 ($P = 0.407$). However, the production of PGE_2 was different from day 9 and day 16 combined versus day 12 and day 14 combined ($P < 0.001$). The pattern of PGD_2 synthesis was similar to that of PGE_2 ; production was greatest on day 14 (8550 ± 1241 c.p.m. versus 200 ± 1388 , 3384 ± 1388 and 1746 ± 1133 c.p.m. on days 9, 12 and 16, respectively) (day 9 versus day 16, $P = 0.439$; day 12 versus day 14, $P = 0.028$; day 9 and day 16 versus day 12 and day 14, $P < 0.001$). However, secretion of PGD_2 was lower than PGE_2 since PGD_2 represented $< 14\%$ of the total radioactive cyclooxygenase, whereas PGE_2 represented 33% on day 12. Luminal cells produced large amounts of the breakdown product of $\text{PGF}_{2\alpha}$, 13,14-dihydro-15-keto $\text{PGF}_{2\alpha}$ (25–50% of the total metabolites depending on the day of the oestrous cycle). These results indicate the presence of a fully active prostaglandin dehydrogenase enzyme in these cells. The synthesis of 13,14-dihydro-15-keto $\text{PGF}_{2\alpha}$, which represented 52% of the total metabolites on day 9, increased on day 12 and day 14 (13240 ± 3377 , 21420 ± 3377 and 22300 ± 3021 c.p.m. for days 9, 12 and 14, respectively). 13,14-dihydro-15-keto $\text{PGF}_{2\alpha}$ was produced in smaller amounts by cells from day 16 (6800 ± 2757 c.p.m.) (day 9 versus day 16, $P = 0.099$; day 12 versus day 14, $P = 0.829$; day 9 and day 16 versus day 12 and day 14, $P < 0.001$; day 16 versus day 9, day 12 and day 14, $P < 0.001$). Luminal cells also produced 13,14-dihydro-15-keto PGE_2 ,

the main dehydrogenate metabolite of PGE_2 . However, production of this metabolite was sevenfold lower than that of 13,14-dihydro-15-keto $\text{PGF}_{2\alpha}$. The synthesis of 13,14-dihydro-15-keto PGE_2 by cells from day 16 was lower compared with that on days 9, 12 and 14 ($P = 0.006$). The cellular concentration of COX-2 was correlated with the production of PGE_2 ($R = 0.550$; $P < 0.001$) and PGD_2 ($R = 0.630$; $P < 0.001$), whereas there was no relationship between COX-2 concentrations and the other prostaglandins.

Comparison of prostaglandin production by luminal cells from pregnant and non-pregnant ewes

The synthesis of $\text{PGF}_{2\alpha}$, PGE_2 , PGD_2 and 13,14-dihydro-15-keto $\text{PGF}_{2\alpha}$ by luminal cells from pregnant and non-pregnant ewes is shown (Figs 5–8). The effect of reproductive status was examined by day using one-way ANOVA. There was no difference in prostaglandin production between pregnant and non-pregnant ewes ($\text{PGF}_{2\alpha}$, $P = 0.468$; PGE_2 , $P = 0.753$; PGD_2 , $P = 0.565$; 13,14-dihydro-15-keto $\text{PGF}_{2\alpha}$, $P = 0.763$). For each metabolite, there was no interaction between reproductive status and day.

Discussion

The present study supports the study of Charpigny *et al.* (1997a) which showed a marked increase in endometrial COX-2 on day 12 of the oestrous cycle. The present study also demonstrated that the COX-2 protein is only detectable in luminal epithelial cells using western blot analysis. This confirms earlier findings using immunohistochemical techniques (Charpigny *et al.*, 1997a). The concentration of

Fig. 5. PGF_{2α} synthesis in luminal cells from pregnant (■) and non-pregnant (□) ewes at days 9, 12, 14 and 16 of the oestrous cycle or pregnancy. Bars represent least square mean values $\pm$ SE (day 9, $P = 0.329$; day 12, $P = 0.268$; day 14, $P = 0.304$; day 16, $P = 0.285$).

Fig. 7. PGD₂ synthesis in luminal cells from pregnant (■) and non-pregnant (□) ewes on days 9, 12, 14 and 16 of the oestrous cycle or pregnancy. Bars represent least square mean values $\pm$ SE (day 9, $P = 0.543$; day 12, $P = 0.744$; day 14, $P = 0.154$; day 16, $P = 0.118$).

Fig. 6. PGE₂ synthesis in luminal cells from pregnant (■) and non-pregnant (□) ewes on days 9, 12, 14 and 16 of the oestrous cycle or pregnancy. Bars represent least square mean values $\pm$ SE (day 9, $P = 0.054$; day 12, $P = 0.387$; day 14, $P = 0.397$; day 16, $P = 0.458$).

Fig. 8. 13,14-dihydro-15-keto PGF_{2α} synthesis in luminal cells from pregnant (■) and non-pregnant (□) ewes on days 9, 12, 14 and 16 of the oestrous cycle or pregnancy. Bars represent least square mean values $\pm$ SE (day 9, $P = 0.890$; day 12, $P = 0.839$; day 14, $P = 0.811$; day 16, $P = 0.245$).

COX-2 was extremely high since it reached 110 ng μ g⁻¹ cellular protein on day 14. It was demonstrated that endometrial luminal cells are mainly responsible for the marked increase in uterine prostaglandin production in the late luteal phase. Therefore, high concentrations of COX-2 may be required for extensive prostaglandin synthesis. On the basis of enzyme content, the increase in prostaglandin synthesis appears to rely mostly on COX-2 upregulation since COX-1 remained at a low concentration. As reported by Charpigny *et al.* (1997a), the present study indicates that the presence of a conceptus prevents the downregulation of COX-2 expression normally occurring on day 16 of the oestrous cycle. Asselin *et al.* (1997c) showed that IFN- τ upregulated COX-2 *in vitro* suggesting that it could be involved in the maintenance of COX-2 expression *in vivo*. However, progesterone could also be involved in the maintenance of COX-2 expression during early pregnancy since it was demonstrated that COX-2 expression was positively regulated by progesterone (Charpigny *et al.*, 1997a; Wu *et al.*, 1997).

The present study showed that the luminal cells produce the five primary prostaglandins: prostacyclin and

thromboxane A₂, measured by their spontaneous breakdown products 6-keto PGF_{1 α} and thromboxane B₂, respectively, PGF_{2 α} , PGE₂ and PGD₂. The synthesis of 6-keto PGF_{1 α} by ovine uterine tissue was reported by Marcus (1981), but despite speculation regarding a putative luteotrophic activity of the molecule, the function of uterine prostacyclin remains unknown. The present study is the first to report that ovine endometrial cells produced thromboxane and a significant amount of PGD₂. In the central nervous system, PGD₂ is known to be an endogenous sleep-inducing substance, but the function of PGD₂ in reproduction is poorly documented. In sheep, PGD₂ is the most potent primary prostaglandin in increasing endometrial and myometrial blood flow when infused into the uterine lumen (Schramm *et al.*, 1984). It is suggested that the endogenous production of PGD₂ contributes to the regulation of uterine blood flow which increases late in the luteal phase.

Total prostaglandin production by the luminal cells increased as COX-2 concentration increased and was at its lowest value on day 9 when COX-2 was not expressed. This finding indicates that the regulation of prostaglandin synthesis is closely linked to COX-2 expression. However,

only PGE₂ and PGD₂ secretion were affected by the upregulation of COX-2. In contrast, the variation in total production of PGF_{2α} (PGF_{2α} plus 13,14-dihydro-15-keto PGF_{2α}) was not correlated with COX-2 cellular content. Total production of PGF_{2α} increased on days 9 and 16, whereas COX-2 was not expressed on day 9 and was expressed in lower amounts on day 16 compared with days 12–14. These results indicate that COX-1 could be the main source of PGF_{2α} synthesis rather than COX-2. However, the findings should be interpreted carefully since prostaglandin synthesis from exogenous [³H]arachidonic acid may not reflect endogenous synthetic capabilities. Since COX-1 and COX-2 are differentially localized in the cell (Morita *et al.*, 1995; Smith *et al.*, 1996) the availability of exogenous arachidonic acid could be different for the two isoforms and may lead to differences in the profiles of radioactive and non-radioactive release. In other species, there is some evidence that COX-2 is involved in the release of PGF_{2α} from the uterus (Naderali and Poyser, 1996). Mice lacking the gene encoding the receptor for PGF_{2α} fail to undergo parturition (Sugimoto *et al.*, 1997), whereas mice deficient in COX-1 deliver offspring normally (Lagenbach *et al.*, 1995). This finding indicates that COX-2 is responsible for the PGF_{2α} synthesis required for parturition. The fact that *in vitro* production of PGF_{2α} was not correlated with COX-2 concentration could be due to a PGF_{2α} synthetic pathway that was not functional *in vitro* or was not switched on. A defective prostaglandin F synthase would result in an increase in the conversion of the COX-2 product, PGH₂, to other prostaglandins such as PGE₂ at the expense of PGF_{2α}. An alternative mechanism to explain the different relationship between COX-2 content and the synthesis of PGF_{2α} and PGE₂ may involve the enzymatic properties of PGF_{2α} and PGE₂ synthases. The affinity of PGE₂ synthase for PGH₂ was reported to be 10 to 30 times that of PGF_{2α} synthase for PGH₂ (K_m for PGE₂ synthase: 150–300 μmol l⁻¹; K_m for PGF_{2α} synthase: 10–25 μmol l⁻¹) (Urade *et al.*, 1995). Consequently, an important increase in the availability of PGH₂ after the increase in COX-2 would result in preferential PGE₂ production. It is also possible that the important PGF_{2α} output observed *in vivo* may be linked to a source of arachidonic acid directed specifically into the PGF_{2α}-forming pathway, which could not be evidenced *in vitro*.

In the present study, PGF_{2α} remained at similar concentrations from day 9 to day 14, whereas COX-2 expression was highly stimulated during this period. Cells appeared to maintain a steady PGF_{2α} output by means of degradative activity since 13,14-dihydro-15-keto PGF_{2α} was released in large amounts. On day 16, PGF_{2α} was released in greater amounts than on days 9–14, whereas the 13,14-dihydro-15-keto PGF_{2α} output was reduced compared with days 9–14.

The increase in PGE₂ synthesis on days 12–14 observed in this study is in agreement with experiments *in vivo* and *in vitro* (Ellinwood *et al.*, 1979; Marcus, 1981; Lacroix and Kann, 1983). The total production of PGF_{2α} (PGF_{2α} plus 13,14-dihydro-15-keto PGF_{2α}) was equivalent to that of PGE₂. This result is in accordance with the findings of Lacroix *et al.* (1983) who reported that PGF_{2α} and PGE₂ were produced in equivalent amounts by the luminal side of the ovine

endometrium *in vitro*. However, the results of the present study are in contrast to those obtained from monolayers of cultured epithelial cells from ovine (Cherny and Findlay, 1990) and bovine endometrium (Fortier *et al.*, 1988) in which PGF_{2α} secretion was two to ten times that of PGE₂. This may be explained by differences in culture techniques. It is speculated that the short-term culture system used in the present study reflects better the physiology of endometrial cells compared with the long-term culture of monolayers. The expression of endometrial COX-2 was induced by steroid hormones and appears to be regulated by other factors such as IFN-τ (Asselin *et al.*, 1997c) and oxytocin (Asselin *et al.*, 1997b; Burns *et al.*, 1997). *In vitro*, it is difficult to reproduce the combined effect of factors regulating COX-2 expression and it is likely that in long-term culture the regulation of COX-2 expression was altered by cell culture conditions.

Many studies of endometrial prostaglandin metabolism have focused on the mechanism of the pulsatile secretion of PGF_{2α}. Luteolysis was induced by PGF_{2α} release in response to oxytocin (Fairclough *et al.*, 1980) and the timing of the first PGF_{2α} pulse was closely associated with endometrial oxytocin receptor concentration which increases progressively in the late luteal phase (Roberts *et al.*, 1976; Sheldrick and Flint, 1985). The present study showed that COX-2 concentrations in the luminal epithelial cells increased at the time of, or just before, the start of pulsatile release of PGF_{2α} (Thorburn *et al.*, 1973). This indicates that the increase in enzyme concentration is a prerequisite for the important production of prostaglandins occurring during the late luteal phase. However, the results of the present study show that the increase in prostaglandin synthesizing capacity results in a concomitant increase in PGE₂ production indicating that COX-2-dependent prostaglandin production may have a luteotrophic effect. The results are in agreement with those of Asselin *et al.* (1997c) who reported that the stimulation of COX-2 expression resulted in a parallel increase of PGE₂ production by bovine endometrial cells. Other studies showed that PGE₂ is produced by the ovine uterus (Lewis *et al.*, 1978; Ellinwood *et al.*, 1979; Marcus, 1981; Lacroix and Kann, 1983). It was proposed (Silvia *et al.*, 1984) that PGE₂ has a role in preventing luteolysis as it delayed luteolysis when infused into the uterus (Pratt *et al.*, 1979) or the ovarian artery (Henderson *et al.*, 1977). However, additional roles have been reported for PGE₂ in association with cellular differentiation. PGE₂ is involved in changes in vascular permeability and stromal decidualization in rodents (Kennedy, 1983). Uterine PGE₂ is also a potent mediator of leucocyte function (Kelly, 1994) and inhibits the lysis of trophoblast cells by natural killer cells (Parhar *et al.*, 1989; Bergeron *et al.*, 1997).

The results of the present study indicate that the endometrial epithelium can convert PGF_{2α} and PGE₂ to their inactive stable metabolites 13,14-dihydro-15-keto PGF_{2α} and 13,14-dihydro-15-keto PGE₂. In rodents and primates, the decidual cells originating from the stromal compartment of the endometrium were generally thought to be responsible for the expression of uterine prostaglandin dehydrogenase during pregnancy. However, in the non-pregnant human uterus, prostaglandin dehydrogenase activity is localized

primarily in epithelial endometrium (Casey *et al.*, 1980). Bracken *et al.* (1997) reported that prostaglandin dehydrogenase mRNA transcripts were found in stromal and epithelial cells of cyclic guinea-pig uterus.

The observation that luminal cells inactivate $\text{PGF}_{2\alpha}$ at a higher rate than PGE_2 raises questions about the enzyme involved. A low K_m value of $\text{PGF}_{2\alpha}$ for the prostaglandin dehydrogenase, as reported in the ovine endometrium (Keirse *et al.*, 1977), could explain the greater production of 13,14-dihydro-15-keto $\text{PGF}_{2\alpha}$ than 13,14-dihydro-15-keto PGE_2 . Another possibility is that $\text{PGF}_{2\alpha}$ and PGE_2 are converted by two different prostaglandin dehydrogenase enzymes. This hypothesis was proposed to explain the differential catabolism of $\text{PGF}_{2\alpha}$ and PGE_2 in the rat uterus (Franchi *et al.*, 1985). Lee and Levine (1975) identified two types of prostaglandin dehydrogenase with distinct cofactor requirements and substrate specificities. The apparent K_m values of PGE_2 and $\text{PGF}_{2\alpha}$ for type I were 10 and 25 μM , respectively, and were 200 and 25 μM , respectively, for type II (Hansen, 1976). If such an enzyme is present in the ovine endometrium, the rather high K_m value of PGE_2 for the type II enzyme could explain the lower production rate of 13,14-dihydro-15-keto PGE_2 . Type II prostaglandin dehydrogenase has been found in many tissues (for a review, see Hansen, 1976) including uterus and placenta (Westbrook *et al.*, 1977; Niimura and Ishida, 1978). The results of the present study indicate that the prostaglandin dehydrogenase activity is regulated during the oestrous cycle since 13,14-dihydro-15-keto $\text{PGF}_{2\alpha}$ was lower on day 16 compared with its activity on days 9–14. Franchi *et al.* (1985) and Bracken *et al.* (1997) reported that prostaglandin dehydrogenase activity increased under oestrogenic stimulation. In contrast, the results of the present study indicate prostaglandin dehydrogenase activity was low when the ratio of oestradiol:progesterone is almost at the maximum.

The pattern of prostaglandin synthesis in cells from pregnant ewes was comparable to the pattern observed in cells from non-pregnant ewes, in agreement with other reports (Ellinwood *et al.*, 1979; Lacroix and Kann, 1983). It is likely that the similar concentrations of cyclooxygenases between pregnant and non-pregnant animals accounted for the similar amounts of prostaglandin synthesis. The results are in agreement with the report of Salamonsen *et al.* (1991) in which no differences in prostaglandin synthase mRNA were observed between early pregnant and cyclic ewes. It is apparent that the ability of the endometrium to produce prostaglandins develops similarly in early pregnant and non-pregnant ewes and numerous studies have demonstrated that the overall production of prostaglandins is similar in early pregnancy and the oestrous cycle (Ellinwood *et al.*, 1979; Lacroix and Kann, 1983; Zarco *et al.*, 1988). However, the main difference observed in prostaglandin synthesis was the pattern of $\text{PGF}_{2\alpha}$ release, which was pulsatile in cyclic ewes and constant in early pregnant ewes (Zarco *et al.*, 1988). The results of the present study in which the $\text{PGF}_{2\alpha}$ output by cells from day 16 pregnant ewes was lower than that from day 16 cyclic ewes may reflect the situation *in vivo*.

In summary, this study demonstrates that the marked increase in endometrial COX-2 results in a parallel increase in prostaglandin synthesis which is limited to PGE_2 and

PGD_2 since, until day 14, the basal production of $\text{PGF}_{2\alpha}$ is maintained at steady state by conversion towards 13,14-dihydro-15-keto $\text{PGF}_{2\alpha}$.

The authors thank Guenahel Danet-Desnoyers for critical review of this manuscript, and Aline Solari and Marie Madeleine Mialon for help with the statistical analysis.

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