Evaluation of the contribution of cyclooxygenase 1 and cyclooxygenase 2 to the production of PGE₂ and PGF_{2α} in epithelial cells from bovine endometrium

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In ruminants, the production of prostaglandins by the endometrium is critical for recognition of pregnancy. In the absence of an embryonic signal, luteolytic pulses of PGF_{2α} are released by the uterus. In contrast, the presence of a viable conceptus reduces the production of PGF_{2α} relative to PGE₂ and prevents luteolysis through the release of trophoblastic interferon (IFN-τ). Initially, it was thought that epithelial and stromal endometrial cells were specialized in the production of a single type of prostaglandin. However, purified cell populations of both types of cell can produce PGF_{2α} and PGE₂; therefore, selective production of PGF_{2α} and PGE₂ must be regulated within each type of cell. Two distinct prostaglandin synthases, cyclooxygenase 1 and cyclooxygenase 2, are involved in prostaglandin production and each may catalyse the production of a different prostaglandin. This possibility was investigated in cultured epithelial cells from bovine endometrium. Cells were

treated with oxytocin or arachidonic acid, and expression of cyclooxygenase 1 and cyclooxygenase 2 proteins was monitored over time and correlated with prostaglandin accumulation. Cells were also treated with increasing doses of inhibitors of cyclooxygenase 1 or cyclooxygenase 2 (non-steroidal anti-inflammatory drugs; NSAIDs) with or without arachidonic acid or oxytocin: flurbiprofen (0–50 μmol l⁻¹) was used as a non-selective inhibitor; valeryl salicylate (0–500 μmol l⁻¹) was used as a cyclooxygenase 1 inhibitor and NS-398 (0–1 μmol l⁻¹) was used as a cyclooxygenase 2 inhibitor. After stimulation with arachidonic acid or oxytocin, prostaglandin production and expression of cyclooxygenase 2 protein were increased. All inhibitors were able to block basal and stimulated prostaglandin production. These results indicate that in endometrium most, if not all, prostaglandin production is probably processed through cyclooxygenase 2.

Introduction

Prostaglandins are important regulators of reproductive events including ovulation, implantation, parturition, luteolysis and recognition of pregnancy (Poyser, 1995; Dubois *et al.*, 1998). In ruminants, PGF_{2α} originating from the endometrium is responsible for luteolysis (McCracken *et al.*, 1999). Exogenous PGF_{2α} can be used to terminate pregnancy and to initiate a new oestrous cycle for synchronization of ovulation in cyclic animals (Horton and Poyser, 1976; McCracken *et al.*, 1999). Although it is less clearly established, PGE₂ is thought to exert actions opposite to PGF_{2α} to favour establishment of pregnancy and may have a luteoprotective action that is either luteotrophic or antiluteolytic (Pratt *et al.*, 1977; Magness *et al.*, 1981). PGE₂ also modulates the immune

system to prevent rejection of the embryo allograft (Lala, 1990).

In bovine endometrium, epithelial and stromal cells have specific morphological and functional properties. Epithelial cells preferentially produce PGF_{2α}, whereas stromal cells produce mainly PGE₂ (Fortier *et al.*, 1988; Kim and Fortier, 1995; Asselin *et al.*, 1996, 1997a,b,c). In bovine endometrial cells in primary culture, PGF_{2α} content is increased after stimulation with oxytocin in epithelial cells only (Kim and Fortier, 1995; Asselin *et al.*, 1996). In contrast, exposure to interferon tau (IFN-τ), the pregnancy recognition signal produced by the embryo in ruminants, increases PGE₂ production by both epithelial and stromal cells when used at high physiological concentrations (Asselin *et al.*, 1997a). This stimulation is such that it changes the relative production of prostaglandin in favour of PGE₂ in epithelial cells. In stromal cells, PGE₂ production is also increased by IFN-τ, maintaining the preferential production of PGE₂ in these cells (Asselin *et al.*, 1997a).

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Cyclooxygenase proteins are rate-limiting enzymes for the conversion of arachidonic acid into PGH_2 , the common precursor of all prostaglandins. There are two isoforms of cyclooxygenase: cyclooxygenase 1 and cyclooxygenase 2. The constitutive isoform, cyclooxygenase 1, is expressed in most nucleated cells, whereas the inducible cyclooxygenase 2 isoform plays a role in various pathological and some physiological conditions (Smith *et al.*, 1996, 2000). The same authors proposed that the two different cyclooxygenase isoforms could be associated with distinct pools of arachidonic acid and different downstream enzymes (Smith *et al.*, 1996, 2000). Accordingly, it has been shown that different prostaglandin isomerases are functionally associated with cyclooxygenase 1 and cyclooxygenase 2 when transfected into HEK-293 cells (Murakami *et al.*, 2000; Tanioka *et al.*, 2000). The activity of cyclooxygenase can be blocked by non-steroidal anti-inflammatory drugs (NSAIDs) (Poyser, 1995). Selective inhibitors for the cyclooxygenase 2 isoform have recently been developed. In sheep, cyclooxygenase 1 is expressed at a steady state during the oestrous cycle and in early pregnancy (Charpigny *et al.*, 1997). During the oestrous cycle in sheep, cyclooxygenase 2 is expressed transiently from day 12 to day 15 and expression decreases thereafter, whereas in early pregnancy, expression is first detected on day 12 and remains high until day 17, when it decreases progressively until day 25 (Charpigny *et al.*, 1997). Arosh *et al.* (2002) demonstrated that during the bovine oestrous cycle, cyclooxygenase 1 is not expressed either at the mRNA or at the protein level, but that cyclooxygenase 2 is expressed throughout the cycle with maximal expression between day 16 and day 18. *In vivo*, cyclooxygenase 1 is localized in both epithelial and stromal cells of sheep endometrium, and cyclooxygenase 2 is localized in the luminal epithelium and in the superficial glands (Charpigny *et al.*, 1997). In primary cultures of bovine endometrial cells, northern blot analysis demonstrated that expression of cyclooxygenase 1 mRNA is fairly high in epithelial and stromal cells, whereas expression of cyclooxygenase 2 mRNA is very low under non-stimulated conditions in both types of cell (Asselin *et al.*, 1997c). Asselin *et al.* (1997b) reported an increase in expression of cyclooxygenase 2 mRNA and $\text{PGF}_{2\alpha}$ production after stimulation with oxytocin. In contrast, a similar increase in expression of cyclooxygenase 2 mRNA was observed with increased PGE_2 production after stimulation with $\text{IFN-}\tau$ (Asselin *et al.*, 1997a,c). Expression of cyclooxygenase 1 was not affected by either treatment; cyclooxygenase 1 and cyclooxygenase 2 proteins were not studied (Asselin *et al.*, 1997b,c).

The aim of the present study was to determine whether preferential production of PGE_2 or $\text{PGF}_{2\alpha}$ in endometrial epithelial cells could be attributed to a specific cyclooxygenase isoform. Treatment with oxytocin and arachidonic acid, both of which stimulate

the production of prostaglandin, was used in the presence and absence of NSAIDs to inhibit cyclooxygenase 1 and cyclooxygenase 2.

Materials and Methods

Materials

Tissue culture plates were purchased from Sarstedt (Newton, NC) and fetal bovine serum was obtained from Wisent Inc. (Saint-Bruno, QC). RPMI-1640 was obtained from ICN Biomedicals (Aurora, OH). Cyclooxygenase 1 and cyclooxygenase 2 antibodies were kindly provided by S. Kargman (Merck Frosst, Kirkland, QC). Tracers for PGE_2 and $\text{PGF}_{2\alpha}$ used in the enzyme immunoassay and inhibitors (flurbiprofen, NS-398 and valeryl salicylate) were purchased from Cayman Chemical (Ann Arbor, MI). The goat anti-rabbit and the goat anti-mouse antibodies conjugated to horseradish peroxidase were obtained from Jackson Immunoresearch Laboratories (West Grove, PA). Renaissance western blot chemiluminescence reagent was purchased from Perkin-Elmer (Boston, MA). Oxytocin, arachidonic acid and anti- β -actin antibody were obtained from Sigma (St Louis, MO). Hyperfilm ECL was purchased from Amersham Pharmacia Biotech (Baie d'Urfé, QC).

Isolation and culture of epithelial cells

Bovine uteri were collected from an abattoir within 15 min of death, and the physiological status of the tissue was estimated by examination of ovarian morphology (Arosh *et al.*, 2002). Uteri were transported on ice to the laboratory and dissected in a laminar flow hood. Epithelial cells were isolated by selective digestion with trypsin as described by Fortier *et al.* (1988) and grown in RPMI-1640 supplemented with 10% FBS-DC (depleted of steroids by dextran-charcoal extraction) at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were cultured in 24-well plates; the medium was changed every 2 days until the cells were used. Confluence of epithelial cells occurred after 6–10 days in culture. A total of five early-cycle uteri (days 1–5 determined as described by Arosh *et al.* (2002)) was used.

Experimental protocol

After cells reached confluence, the medium was replaced with 1.0 ml fresh serum-free RPMI-1640 containing stimulators or increasing doses of inhibitors or both (arachidonic acid: $10\text{ }\mu\text{mol l}^{-1}$; oxytocin: $10^{-7}\text{ mol l}^{-1}$; flurbiprofen: $0\text{--}50\text{ }\mu\text{mol l}^{-1}$; valeryl salicylate: $0\text{--}500\text{ }\mu\text{mol l}^{-1}$; NS-398: $0\text{--}1\text{ }\mu\text{mol l}^{-1}$). According to the manufacturer's indications, flurbiprofen is a non-selective inhibitor, with a preferential affinity for cyclooxygenase 1 at $0.04\text{ }\mu\text{mol l}^{-1}$ and for cyclooxygenase 2 at $0.51\text{ }\mu\text{mol l}^{-1}$. Valeryl salicylate, another

non-selective inhibitor (IC_{50} value: 0.8 mmol l^{-1} for cyclooxygenase 1 and 15 mmol l^{-1} for cyclooxygenase 2) was used at concentrations ($1\text{--}500 \text{ }\mu\text{mol l}^{-1}$) close to the cyclooxygenase 1 IC_{50} value, and NS-398 was used as a cyclooxygenase 2 specific inhibitor (IC_{50} value: $220 \text{ }\mu\text{mol l}^{-1}$ for cyclooxygenase 1 and $0.15 \text{ }\mu\text{mol l}^{-1}$ for cyclooxygenase 2). Each treatment was carried out in triplicate. The cells were incubated for 24 h, except for the time-course experiments, in which supernatant was recovered and cells were lysed at each time point indicated. For all experiments, at the end of the incubation period the culture medium was recovered for prostaglandin measurement and was stored at -20°C until further processing. For protein extraction, the cells were lysed with $200 \text{ }\mu\text{l}$ lysis buffer ($10 \text{ mmol Tris-HCl l}^{-1}$, pH 7.4; 1% (w/v) SDS; $1 \text{ mmol dithiothreitol l}^{-1}$; and $1 \text{ mmol phenylmethylsulphonylfluoride l}^{-1}$ (PMSF)) and extraction was performed immediately.

Protein extraction

Protein extraction and measurement were performed as described by Chapdelaine *et al.* (2001). Protein samples were suspended in $30 \text{ }\mu\text{l}$ SDS-PAGE loading buffer ($0.06 \text{ mol Tris-HCl l}^{-1}$, pH 6.8; 1% (w/v) SDS; 1% (v/v) 2-mercaptoethanol; 10% (v/v) glycerol; and 0.025% (w/v) bromophenol blue) and boiled for 3 min. Protein content was estimated using $1 \text{ }\mu\text{l}$ of the sample.

Western blot analysis

Aliquots ($5 \text{ }\mu\text{g}$) of total protein were loaded in each lane and subjected to electrophoresis through 10% (w/v) SDS-polyacrylamide gels followed by electrotransfer on to a nitro-cellulose membrane. Prestained protein markers were used as molecular weight standards for each analysis. After staining with Ponceau Red to ensure that the same amount of protein was transferred on to the membrane, blocking was done in 5% (w/v) fat-free dry milk powder in PBS and 0.05% (v/v) Tween-20 (PBS-Tween) overnight at 4°C . The membrane was incubated with the antibody raised against cyclooxygenase 2 (lot no. 243; Merck Frosst) or cyclooxygenase 1 (lot no. 241; Merck Frosst; dilution 1 in 3000), or β -actin (dilution 1 in 5000 in 2% (w/v) fat-free dry milk powder in PBS-Tween) for 1 h at room temperature. Washings were done for 30 min in PBS-Tween. The second antibody, goat anti-rabbit (cyclooxygenase 1 or cyclooxygenase 2 analysis) or goat anti-mouse (β -actin analysis) conjugated to horseradish peroxidase (dilution 1 in 10 000 in 2% (w/v) fat-free dry milk powder in PBS-Tween) was then incubated for 45 min at room temperature. The membrane was washed for another 30 min in PBS-Tween. Bands were revealed by addition of a chemiluminescent substrate applied according to the manufacturer's instructions (Renaissance; Perkin-Elmer). The blots were exposed

to Hyperfilm ECL with intensifying screen. The bands were quantified by Biolmage Visage 110s from Genomic Solutions Inc. (Ann Harbor, MI).

ELISA for prostaglandins

An enzyme immunoassay was used for measurement of PGE_2 and $\text{PGF}_{2\alpha}$, using acetylcholinesterase-linked prostaglandin tracers as described by Asselin *et al.* (1996). Fully characterized rabbit anti- PGE_2 (Evans *et al.*, 1982; Asselin *et al.*, 1996) and sheep anti- $\text{PGF}_{2\alpha}$ (Bio Quant, Ann Arbor, MI) were used. The inter- and intra-assay coefficients of variation ($n = 12$) were 16 and 10%, respectively.

Statistical analyses

Data for prostaglandin concentrations are presented as the mean $\pm$ SEM of ratio of protein: β -actin or percentage of control or percentage of content at time 0. The data were treated by analysis of variance using Super ANOVA Software (Abacus Concepts Inc., Berkeley, CA). Sources of variation included experiments, treatments and their interactions. Individual comparison of means was made using Student-Newman-Keuls test. Independent variables were the concentration of inhibitor or time of stimulation, and the dependent variables were the amount of prostaglandins produced or the amount of cyclooxygenase proteins. Differences were considered significant at $P < 0.05$.

Results

Expression of cyclooxygenase 1 and cyclooxygenase 2 protein in relation to $\text{PGF}_{2\alpha}$ and PGE_2 production

Expression of cyclooxygenase 1 and cyclooxygenase 2 proteins was measured by western blot analysis after treatment of epithelial cells with optimal concentrations of oxytocin (Fig. 1) or arachidonic acid (Fig. 2), both of which are stimulators of prostaglandin production. Expression of cyclooxygenase 1 protein was not significantly affected after treatment with oxytocin ($10^{-7} \text{ mol l}^{-1}$; Fig. 1a) or arachidonic acid ($10 \text{ }\mu\text{mol l}^{-1}$; Fig. 2a). In contrast, expression of cyclooxygenase 2 protein was minimal under control conditions and was strongly stimulated by the two agents. Under oxytocin stimulation, the pattern of expression is indicative of induction at 9 and 12 h (Fig. 1b), whereas with arachidonic acid, expression of cyclooxygenase 2 protein was induced maximally at 12 h after treatment (Fig. 2b; $P < 0.05$).

The time courses of prostaglandin production after treatment with oxytocin (Fig. 1c) or arachidonic acid (Fig. 2c) show that both agents stimulate PGE_2 and $\text{PGF}_{2\alpha}$ accumulation (Table 1). The increase in expression of cyclooxygenase 2 protein occurred before the increase

Fig. 1. Expression of cyclooxygenase 1 and 2 proteins in relation to prostaglandin production in the presence of oxytocin. Primary bovine epithelial cells were grown to confluence and treated with oxytocin (10^{-7} mol l^{-1}) for time periods ranging from 0 h to 24 h. The supernatant of each well was collected for prostaglandin estimation by ELISA (in triplicate). Proteins were extracted at each time point and western blot analysis was performed. (a) Expression of cyclooxygenase 1 protein. (b) Expression of cyclooxygenase 2 protein. (c) Amounts of PGE₂ (□) and PGF_{2α} (■). The blots were exposed for 20 s. Representative blots of one experiment are presented. Results are expressed as mean $\pm$ SEM of four different experiments. ^{a-b}Columns with different letters are significantly different ($P < 0.05$).

Fig. 2. Expression of cyclooxygenase 1 and 2 proteins in relation to prostaglandin production in the presence of arachidonic acid. Primary bovine epithelial cells were grown to confluence and treated with arachidonic acid ($10 \mu\text{mol } l^{-1}$) for time periods ranging from 0 h to 24 h. The supernatant of each well was collected for prostaglandin estimation by ELISA (in triplicate). Proteins were extracted at each time point and western blot analysis was performed. (a) Expression of cyclooxygenase 1 protein. (b) Expression of cyclooxygenase 2 protein. (c) Amounts of PGE₂ (□) and PGF_{2α} (■). The blots were exposed for 20 s. Representative blots of one experiment are presented. Results are expressed as mean $\pm$ SEM of four different experiments. ^{ab}Columns with different letters are significantly different ($P < 0.05$).

Table 1. Prostaglandin accumulation in bovine epithelial cells in culture

Condition	PGE ₂ (ng ml ⁻¹)	PGF _{2α} (ng ml ⁻¹)
0 h	0.33 ± 0.19 ^a	0.99 ± 0.58 ^a
24 h	2.42 ± 0.16 ^a	6.49 ± 0.41 ^a
24 h + arachidonic acid	63.71 ± 16.46 ^b	189.47 ± 42.62 ^b
24 h + oxytocin	11.07 ± 2.21 ^c	90.81 ± 17.16 ^c
Flurbiprofen	0.26 ± 0.11 ^a	0.72 ± 0.31 ^a
Valeryl salicylate	0.31 ± 0.06 ^a	1.57 ± 0.28 ^a
NS-398	0.36 ± 0.09 ^a	1.62 ± 0.32 ^a
Oxytocin + flurbiprofen	0.30 ± 0.15 ^a	1.31 ± 0.61 ^a
Oxytocin + valeryl salicylate	0.73 ± 0.42 ^a	1.63 ± 0.48 ^a
Oxytocin + NS-398	1.05 ± 0.18 ^a	2.28 ± 0.79 ^a
Arachidonic acid + flurbiprofen	14.29 ± 1.62 ^a	8.06 ± 0.65 ^a
Arachidonic acid + valeryl salicylate	17.41 ± 5.88 ^a	15.05 ± 3.81 ^a
Arachidonic acid + NS-398	23.54 ± 6.41 ^a	35.68 ± 13.65 ^a

Absolute values (ng ml⁻¹) under control conditions and after treatment with different doses of stimulants or inhibitors (arachidonic acid: 10 μmol l⁻¹; oxytocin: 10⁻⁷ mol l⁻¹; valeryl salicylate: 500 μmol l⁻¹; NS-398: 1 μmol l⁻¹; flurbiprofen: 50 μmol l⁻¹) are shown.

Values are mean ± SEM of three different experiments.

^{a-c}Within each column, values with different superscripts are significantly different ($P < 0.05$).

in prostaglandin production. The accumulation of both prostaglandins was progressive throughout the experimental period up to 24 h, but the difference compared with time 0 was significant only after 24 h ($P < 0.05$). Arachidonic acid induced a 30-fold stimulation of both PGE₂ and PGF_{2α} production, whereas oxytocin preferentially stimulated PGF_{2α} production (14-fold) compared with PGE₂ (fourfold) (Table 1).

Effect of cyclooxygenase inhibitors on selective production of prostaglandins by epithelial cells

The specific contribution of each isoform of cyclooxygenase to the selective production of PGE₂ or PGF_{2α} was evaluated using non-selective and selective inhibitors in the absence and presence of oxytocin or arachidonic acid stimulation. Under non-stimulated conditions (Fig. 3), prostaglandin production was inhibited in a dose-dependent manner by all the NSAIDs tested ($P < 0.05$). Flurbiprofen (Fig. 3a), valeryl salicylate (Fig. 3b) and NS-398 (Fig. 3c) induced similar patterns of inhibition, progressively reducing the production of both PGE₂ and PGF_{2α}. Flurbiprofen and NS-398 inhibited PGE₂ and PGF_{2α} to a similar extent (approximately 90% and 80% inhibition, respectively), whereas valeryl salicylate appears to inhibit PGE₂ more than PGF_{2α} (85% versus 70%, respectively). Flurbiprofen achieved the highest inhibition (90% inhibition at 50 μmol l⁻¹), whereas NS-398 was the most potent inhibitor (80% inhibition at 0.05 μmol l⁻¹).

Fig. 3. Effect of cyclooxygenase inhibitors on selective production of PGE₂ (□) and PGF_{2α} (■) under basal conditions. Primary bovine epithelial cells were grown to confluence and treated for 24 h with (a) flurbiprofen, (b) valeryl salicylate or (c) NS-398. The supernatant of each well was collected for prostaglandin estimation by ELISA (in triplicate). Data are expressed as percentage of control and are mean ± SEM of three different experiments. ^{a-c}Columns with different letters are significantly different ($P < 0.05$).

The NSAIDs induced a dose-dependent inhibition of the production of both prostaglandins in cells treated with oxytocin (Fig. 4) ($P < 0.05$). At low concentrations of

Fig. 4. Effect of cyclooxygenase inhibitors on selective production of PGE₂ (□) and PGF_{2 α} (■) in the presence of oxytocin. Primary bovine epithelial cells were grown to confluence and treated with oxytocin 10 min before treatment with (a) flurbiprofen, (b) valeryl salicylate or (c) NS-398 for 24 h. The supernatant of each well was collected for prostaglandin estimation by ELISA (in triplicate). Data are expressed as percentage of control and are mean $\pm$ SEM of three different experiments. ^{a-d}Columns with different letters are significantly different ($P < 0.05$).

inhibitors, PGF_{2 α} appeared to be reduced preferentially, especially with valeryl salicylate. At higher concentrations, the production of both prostaglandins was

inhibited more completely than was observed without stimulation (reduction of $> 95\%$ for flurbiprofen at $50 \mu\text{mol l}^{-1}$; 93% reduction for valeryl salicylate at $500 \mu\text{mol l}^{-1}$; 85% reduction for NS-398 at $1 \mu\text{mol l}^{-1}$).

A dose-dependent inhibition was also observed in cells treated with arachidonic acid (Fig. 5) ($P < 0.05$). In general, the production of PGF_{2 α} was reduced at lower concentrations and at a greater extent than PGE₂ for all three inhibitors tested. PGE₂ production was inhibited less in arachidonic acid-treated cells than in cells treated with oxytocin or that had not been stimulated (60–75% at maximal concentration).

Discussion

The specific contribution of each cyclooxygenase isoform to the production of prostaglandins was studied *in vitro*. Under non-stimulated conditions, expression of cyclooxygenase 1 protein by endometrial epithelial cells was high, whereas expression of cyclooxygenase 2 was low. Stimulation of the same cells with oxytocin or arachidonic acid at concentrations known to stimulate prostaglandin production increased the amounts of cyclooxygenase 2 but did not affect cyclooxygenase 1. These results support the concept that under basal conditions, PGF_{2 α} , the principal prostaglandin produced by epithelial cells (Fortier *et al.*, 1988; Kim and Fortier, 1995; Asselin *et al.*, 1996, 1997a,b,c), would be produced through constitutively expressed cyclooxygenase 1. The increase in cyclooxygenase 2 in response to stimulators of prostaglandin production supports its identification as an inducible enzyme. This is further supported by the observation that expression of cyclooxygenase 2 protein in response to oxytocin or arachidonic acid treatment closely matched the production of prostaglandins. However, the hypothesis suggesting that the cyclooxygenase 1 pathway could be linked to the production of PGF_{2 α} and the cyclooxygenase 2 pathway to PGE₂ production cannot be supported conclusively by the results of the present study. Both arachidonic acid and oxytocin stimulated the expression of cyclooxygenase 2 protein, but whereas arachidonic acid induced comparable increases in PGF_{2 α} and PGE₂ accumulation, oxytocin stimulated PGF_{2 α} to a greater extent, as was also observed by Asselin *et al.* (1996). The use of selective inhibitors (NS-398 for cyclooxygenase 2; valeryl salicylate at concentrations close to cyclooxygenase 1 IC₅₀ value; and flurbiprofen for cyclooxygenase 1 at low concentrations and cyclooxygenase 2 at high concentrations) did not produce conclusive results. First, all three inhibitors were able to block the production of both PGF_{2 α} and PGE₂ in a dose-dependent way, even under non-stimulated conditions in which cyclooxygenase 1 was the major cyclooxygenase expressed. Second, all three inhibitors reduced prostaglandin production under stimulated conditions, in which cyclooxygenase 2 was presumably responsible for increased prostaglandin production.

These results question the ability of cyclooxygenase 1 to contribute significantly to the production of prostaglandin in the endometrium and the selectivity of the available cyclooxygenase inhibitors in endometrial cells.

It has been reported previously that oxytocin stimulates $\text{PGF}_{2\alpha}$ production in a time- and dose-dependent manner (Asselin *et al.*, 1996), and also expression of cyclooxygenase 2 mRNA and protein (Asselin *et al.*, 1997b; Parent *et al.*, 2003). The results of the present study demonstrate that the increase in prostaglandin production closely followed the induction of expression of cyclooxygenase 2 protein. The increase in expression of cyclooxygenase 2 protein preceded the increase in prostaglandin production; however, it should be noted that accumulation of prostaglandin was measured over time and hence the amounts shown at 24 h represent net production over the entire period of culture. As expression of cyclooxygenase 1 protein did not vary in response to oxytocin treatment, it is assumed that cyclooxygenase 2 is the primary pathway for increased production of prostaglandin under that condition. The same conclusion can be drawn from results with arachidonic acid stimulation. The reported differences in substrate sensitivity of cyclooxygenase 1 and cyclooxygenase 2 with respect to arachidonic acid (Swinney *et al.*, 1997) can be used to predict their relative contributions to the production of $\text{PGF}_{2\alpha}$ and PGE_2 . The concentration of arachidonic acid ($10 \mu\text{mol l}^{-1}$) used in the present study is optimal to study prostaglandin production over time. Indeed, concentrations $< 10 \mu\text{mol l}^{-1}$ did not stimulate prostaglandin production, and higher concentrations induced cell death (results not shown). It has been reported that cyclooxygenase 1 requires concentrations of arachidonic acid $\geq 10 \mu\text{mol l}^{-1}$ to operate, whereas lower concentrations ($< 2.5 \mu\text{mol l}^{-1}$) are oxygenated exclusively by cyclooxygenase 2 (Morita, 2002). In the present study, a progressive increase in prostaglandin production was observed only after induction of cyclooxygenase 2 expression. Thus, it appears that the immunoreactive cyclooxygenase 1 expressed in the bovine endometrial cells was not able to process the exogenous arachidonic acid provided as would be expected from its enzymatic properties (Reddy and Herschman, 1994). The time-course study of cyclooxygenase 2 expression and prostaglandin accumulation showed that expression of cyclooxygenase 2 was a prerequisite for prostaglandin accumulation. As the rate of prostaglandin production followed the expression of cyclooxygenase 2, this pathway appeared more functional in cultured epithelial cells. Naderali and Poyser (1996) concluded that cyclooxygenase 2 was the predominant prostaglandin-forming enzyme in guinea-pig uterus; therefore, it is possible that it may also be the predominant prostaglandin-forming enzyme in bovine endometrium.

If the increased production of prostaglandins in response to oxytocin was mediated by activation of

Fig. 5. Effect of cyclooxygenase inhibitors on selective production of PGE_2 ($\square$) and $\text{PGF}_{2\alpha}$ ($\blacksquare$) in the presence of arachidonic acid. Primary bovine epithelial cells were grown to confluence and treated with oxytocin 10 min before treatment with (a) flurbiprofen, (b) valeryl salicylate or (c) NS-398 for 24 h. The supernatant of each well was collected for prostaglandin estimation by ELISA (in triplicate). Data are expressed as percentage of control and are mean $\pm$ SEM of three different experiments. ^{a-e}Columns with different letters are significantly different ($P < 0.05$).

cyclooxygenase 2 expression, the effect of the cyclooxygenase inhibitors is problematic. The production of $\text{PGF}_{2\alpha}$ appeared more sensitive to inhibition by all three inhibitors at low concentrations, and at high concentrations in the presence of arachidonic acid. The comparable effects of cyclooxygenase 1 and cyclooxygenase 2 inhibitors are strongly indicative of a lack of selectivity in our system. Similar results were observed in enterocytes by Longo *et al.* (1998), who concluded that cyclooxygenase inhibitors produce various responses depending on conditions and cell models. Such differences are also used for clinical management of hypertension with aspirin through differential sensitivity of platelets and endothelial cells.

The high cyclooxygenase 1 expression in endometrial epithelial cells in culture is difficult to explain as Arosh *et al.* (2002) showed that this isoform is not expressed in bovine endometrium during the oestrous cycle. In contrast, in ovine uterus cyclooxygenase 1 is expressed during the oestrous cycle without any modulation of its expression (Charpigny *et al.*, 1997). Even if it is concluded that cells behave differently in culture, the fact that no variation in the expression of cyclooxygenase 1 protein was observed when prostaglandin production was stimulated supports the conclusion that cyclooxygenase 1 does not contribute to the production of prostaglandin in cultured endometrial cells. Indeed, cyclooxygenases are suicide enzymes that disappear as they catalyse the formation of prostaglandin and, thus, the amounts of cyclooxygenase 1 protein should have decreased rapidly in the absence of messenger to replenish the pool. An alternative explanation could be that the cyclooxygenase 1 protein that was measured was present in an inactive form or in an inaccessible location such as occurs when it is located in the nucleus (Morita *et al.*, 1995).

In conclusion, the hypothesis that cyclooxygenase 1 is preferentially linked to the production of $\text{PGF}_{2\alpha}$ and that cyclooxygenase 2 is preferentially linked to PGE_2 production in bovine endometrium cannot be supported by the results of the present study. In addition, the inhibitors tested were non-selective in our system, as all three showed the same pattern of inhibition for both prostaglandins. Cyclooxygenase 1 was present in high amounts and may contribute to the low amounts of prostaglandins produced under basal conditions, but it does not appear to contribute to any increase in prostaglandin production. However, these findings contradict our recent observations *in vivo* showing no expression of cyclooxygenase 1 in bovine endometrium during the oestrous cycle (Arosh *et al.*, 2002). In contrast, expression of cyclooxygenase 2 was low under basal conditions, but could be induced by oxytocin and arachidonic acid, thus increasing prostaglandin production. Together, these results indicate that cyclooxygenase 2 is probably the functional isoform present in bovine endometrium. If this is also true in mice, it could

explain why cyclooxygenase 2 and not cyclooxygenase 1 knockout females show multiple reproductive failures (Lim *et al.*, 1997).

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References

- Arosh JA, Parent J, Chapdelaine P, Sirois J and Fortier MA (2002) Expression of cyclooxygenases 1 and 2 and prostaglandin E synthase in bovine endometrial tissue during the oestrus cycle *Biology of Reproduction* **67** 161–169
- Asselin E, Goff AK, Bergeron H and Fortier MA (1996) Influence of sex steroids on the production of prostaglandin $\text{F}_{2\alpha}$ and E_2 and response to oxytocin in cultured epithelial and stromal cells of the bovine endometrium *Biology of Reproduction* **54** 371–379
- Asselin E, Bazer FW and Fortier MA (1997a) Recombinant ovine and bovine interferons tau regulate prostaglandin production and oxytocin response in cultured bovine endometrial cells *Biology of Reproduction* **56** 402–408
- Asselin E, Drolet P and Fortier MA (1997b) Cellular mechanisms involved during oxytocin-induced prostaglandin $\text{F}_{2\alpha}$ production in endometrial epithelial cells *in vitro*: role of cyclooxygenase-2 *Endocrinology* **138** 4798–4805
- Asselin E, Lacroix D and Fortier MA (1997c) IFN-tau increases PGE_2 production and COX-2 gene expression in the bovine endometrium *in vitro*. *Molecular and Cellular Endocrinology* **132** 117–126
- Chapdelaine P, Vignola K and Fortier MA (2001) Protein estimation directly from SDS-PAGE loading buffer for standardisation of samples from cell lysates or tissue homogenates before western blot analysis *Biotechniques* **31** 478–482
- Charpigny G, Reinaud P, Tamby JP, Creminon C, Martal J, Maclouf J and Guillomot M (1997) Expression of cyclooxygenase-1 and -2 in ovine endometrium during the oestrus cycle and early pregnancy *Endocrinology* **138** 2163–2171
- Dubois RN, Abramson SB, Crofford L, Gupta RA, Simon LS, Van De Putte LB and Lipsky PE (1998) Cyclooxygenase in biology and disease *FASEB Journal* **12** 1063–1073
- Evans CA, Kennedy TG and Challis JR (1982) Gestational changes in prostanoid concentrations in intrauterine tissues and foetal fluids from pregnant sheep, and the relation to prostanoid output *in vitro*. *Biology of Reproduction* **27** 1–11
- Fortier MA, Guilbault LA and Grasso F (1988) Specific properties of epithelial and stromal cells from the endometrium of cows *Journal of Reproduction and Fertility* **83** 239–248
- Horton EW and Poyser NL (1976) Uterine luteolytic hormone: a physiological role for prostaglandin $\text{F}_{2\alpha}$ *Physiological Reviews* **56** 595–651
- Kim JJ and Fortier MA (1995) Cell type specificity and protein kinase C dependency on the stimulation of prostaglandin E_2 and prostaglandin $\text{F}_{2\alpha}$ production by oxytocin and platelet-activating factor in bovine endometrial cells *Journal of Reproduction and Fertility* **103** 239–247
- Lala PK (1990) Interruption of murine pregnancy by activation of antigen-non-specific killer cells in the endometrium with indomethacin, high dose IL-2 or a combination *Research in Immunology* **141** 159–167
- Lim H, Paria BC, Das SK, Dinchuk JE, Langenbach R, Trzaskos JM and Dey SK (1997) Multiple female reproductive failures in cyclooxygenase 2-deficient mice *Cell* **91** 197–208
- Longo WE, Panesar N, Mazuski J and Kaminski DL (1998) Contribution of cyclooxygenase-1 and cyclooxygenase-2 to prostanoid formation by

- human enterocytes stimulated by calcium ionophore and inflammatory agents *Prostaglandins and Other Lipid Mediators* **56** 325–339
- McCracken JA, Custer EE and Lamsa JC** (1999) Luteolysis: a neuroendocrine-mediated event *Physiological Reviews* **79** 263–323
- Magness RR, Huie JM, Hoyer GL, Huecksteadt TP, Reynolds LP, Seperich GJ, Whyson G and Weems CW** (1981) Effect of chronic ipsilateral or contralateral intrauterine infusion of prostaglandin E₂ (PGE₂) on luteal function of unilaterally ovariectomized ewes *Prostaglandins and Medicine* **6** 389–401
- Morita I** (2002) Distinct functions of COX-1 and COX-2 *Prostaglandins and Other Lipid Mediators* **68–69** 165–175
- Morita I, Schindler M, Regier MK, Otto JC, Hori T, DeWitt DL and Smith WL** (1995) Different intracellular locations for prostaglandin endoperoxide H synthase-1 and -2 *Journal of Biological Chemistry* **270** 10 902–10 908
- Murakami M, Naraba H, Tanioka T et al.** (2000) Regulation of prostaglandin E₂ biosynthesis by inducible membrane-associated prostaglandin E₂ synthase that acts in concert with cyclooxygenase-2 *Journal of Biological Chemistry* **275** 32 783–32 792
- Naderali EK and Poyser NL** (1996) Effect of a selective prostaglandin H synthase-2 inhibitor (NS-398) on prostaglandin production by the guinea-pig uterus *Journal of Reproduction and Fertility* **108** 75–80
- Parent J, Villeneuve C, Alexenko AP, Ealy AD and Fortier MA** (2003) Influence of different isoforms of recombinant trophoblastic interferons on prostaglandin production in cultured bovine endometrial cells *Biology of Reproduction* **68** 1035–1043
- Poyser NL** (1995) The control of prostaglandin production by the endometrium in relation to luteolysis and menstruation *Prostaglandins, Leukotrienes and Essential Fatty Acids* **53** 147–195
- Pratt BR, Butcher RL and Inskeep EK** (1977) Antiluteolytic effect of the conceptus and of PGE₂ in ewes *Journal of Animal Science* **45** 784–791
- Reddy ST and Herschman HR** (1994) Ligand-induced prostaglandin synthesis requires expression of the TIS10/PGS-2 prostaglandin synthase gene in murine fibroblasts and macrophages *Journal of Biological Chemistry* **269** 15 473–15 480
- Smith WL, Garavito RM and DeWitt DL** (1996) Prostaglandin endoperoxide H synthases (cyclooxygenases)-1 and -2 *Journal of Biological Chemistry* **271** 33 157–33 160
- Smith WL, DeWitt DL and Garavito RM** (2000) Cyclooxygenases: structural, cellular, and molecular biology *Annual Review of Biochemistry* **69** 145–182
- Swinney DC, Mak AY, Barnett J and Ramesha CS** (1997) Differential allosteric regulation of prostaglandin H synthase 1 and 2 by arachidonic acid *Journal of Biological Chemistry* **272** 12 393–12 398
- Tanioka T, Nakatani Y, Semmyo N, Murakami M and Kudo I** (2000) Molecular identification of cytosolic prostaglandin E₂ synthase that is functionally coupled with cyclooxygenase-1 in immediate prostaglandin E₂ biosynthesis *Journal of Biological Chemistry* **275** 32 775–32 782

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