Differential effects of prostaglandin $F_{2\alpha}$ on *in vitro* luteinized bovine granulosa cells

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Prostaglandins have been implicated in various aspects of ovarian function including ovulation and luteolysis. In this study, the expression and regulation of inducible prostaglandin G/H synthase (PGHS-2) and $PGF_{2\alpha}$ receptors were investigated in bovine granulosa cells at various stages of differentiation. Firstly, the induction of $PGF_{2\alpha}$ receptor mRNA and PGHS-2 mRNA in preovulatory granulosa cells was evaluated. Granulosa cells were collected from preovulatory follicles and cultured for 1, 4, 7 or 10 days. Cells were treated with hCG (10 iu) or with increasing doses of forskolin (0–10 $\mu\text{mol l}^{-1}$) for 24 h. Forskolin increased steady-state concentrations of mRNA for PGHS-2 (> 20-fold) and $PGF_{2\alpha}$ receptor (> 1000-fold) in a dose-dependent fashion. Use of selective protein kinase A inhibitor (H89) reduced both hCG- and forskolin-induced expression of $PGF_{2\alpha}$ receptor mRNA and PGHS-2 mRNA. The hypothesis that luteinized granulosa cells would

acquire $PGF_{2\alpha}$ responsiveness similar to responses to $PGF_{2\alpha}$ observed *in vivo* was also evaluated. Treatment with $PGF_{2\alpha}$ (100 nmol l^{-1}) reduced forskolin-induced expression of $PGF_{2\alpha}$ receptor mRNA on days 4, 7 and 10, but not on day 1 of culture ($n = 3$). Treatment with $PGF_{2\alpha}$ did not change forskolin-induced expression of PGHS-2 mRNA on or before day 4 of culture. In contrast, $PGF_{2\alpha}$ significantly increased PGHS-2 mRNA expression in granulosa cells primed with forskolin for 7 or 10 days. In conclusion, expression of PGHS-2 and $PGF_{2\alpha}$ receptor mRNA is protein kinase A-dependent in preovulatory bovine granulosa cells. Granulosa cells become $PGF_{2\alpha}$ -responsive soon after expression of $PGF_{2\alpha}$ receptor, whereas further differentiation is required before $PGF_{2\alpha}$ induces PGHS-2 mRNA upregulation. These results demonstrate that at least two key transitions are required in $PGF_{2\alpha}$ -induced luteal regression in the mid-cycle corpus luteum.

Introduction

A number of critical events occur in the ovary during the oestrous cycle. The two most marked transitions, ovulation and luteolysis, are primarily due to changes in serum hormone concentrations in most species. The capacity to respond to hormones requires specific intracellular alterations. Preovulatory follicles acquire the capacity to ovulate in response to LH apparently due to the expression of LH receptors in the granulosa cells (Sartori *et al.*, 1998). The corpus luteum acquires the capacity to regress in response to $PGF_{2\alpha}$ (luteolytic capacity) approximately 7 days after the LH surge. Acquisition of luteolytic capacity does not appear to be due to increased expression of $PGF_{2\alpha}$ receptor (Tsai and Wiltbank, 1998). Treatment of cows with $PGF_{2\alpha}$ on day 4 (no luteolytic capacity) or day 11 (luteolytic capacity) caused a similar decrease in luteal vitamin C and luteal concentrations of certain mRNAs, including $PGF_{2\alpha}$ receptor mRNA (Tsai and Wiltbank, 1998). This finding indicates that $PGF_{2\alpha}$ reached the corpus luteum and initiated certain cellular and molecular responses even though luteal regression did not occur in the early corpus luteum.

The action of $PGF_{2\alpha}$ is mediated through binding to its plasma membrane receptor, the G protein-coupled $PGF_{2\alpha}$ receptor. Binding of $PGF_{2\alpha}$ by $PGF_{2\alpha}$ receptor activates phospholipase C, increases free intracellular calcium concentrations and activates protein kinase C (PKC) (Wiltbank *et al.*, 1991). In bovine granulosa cells, $PGF_{2\alpha}$ receptor mRNA content is extremely low (approximately one transcript per cell) before ovulation, but is induced markedly by the LH surge (> 750 transcripts per cell) at 24 h after ovulation (Tsai *et al.*, 1996). A study *in vitro* using bovine granulosa cells demonstrated that expression of $PGF_{2\alpha}$ receptor can be induced by forskolin in a similar time course (Tsai *et al.*, 1996). Treatment *in vivo* with $PGF_{2\alpha}$ decreased expression of $PGF_{2\alpha}$ receptor mRNA in bovine and ovine corpora lutea (Rueda *et al.*, 1995; Juengel *et al.*, 1996; Tsai and Wiltbank, 1998; Tsai *et al.*, 1998). Similarly, $PGF_{2\alpha}$ decreased expression of $PGF_{2\alpha}$ receptor in cultured human granulosa lutein cells (Ristimäki *et al.*, 1997). In contrast, $PGF_{2\alpha}$ receptor expression was upregulated by $PGF_{2\alpha}$ in mouse corpus luteum (Sugatani *et al.*, 1996). It is unclear whether the different effects of $PGF_{2\alpha}$ on the expression of $PGF_{2\alpha}$ receptor are due to species differences or to the different stages at which the cells were treated.

One rate-limiting step in $PGF_{2\alpha}$ biosynthesis is the conversion of arachidonic acid to PGH_2 by prostaglandin

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G/H synthase (PGHS, also known as cyclooxygenase). There are two isoforms of PGHS: the constitutively expressed PGHS-1 and the inducible PGHS-2. PGHS-1 is expressed ubiquitously and is thought to produce prostaglandins for various 'housekeeping' functions, such as platelet aggregation, vasodilatation in the kidney and cytoprotection of gastric mucosa (Langenbach *et al.*, 1995; Smith *et al.*, 1996). In contrast, expression of PGHS-2 is minimal under physiological conditions, but expression is induced rapidly by cytokines, tumour promoters, endotoxins, proinflammatory agents and certain hormones (Herschman, 1994; Smith *et al.*, 1996). The LH surge, via activation of the cAMP–protein kinase A (PKA) signalling cascade, increases PGHS-2 mRNA in granulosa cells from preovulatory follicles and the resulting synthesis of prostaglandins may be critical for the timing of ovulation (Sirois, 1994; Tsai *et al.*, 1996; Sirois and Dore, 1997). Low amounts of PGHS-2 mRNA were expressed in mid-cycle bovine and ovine corpora lutea, but PGF_{2α} markedly increased luteal PGHS-2 mRNA (Tsai and Wiltbank, 1997, 1998). Induction of PGHS-2 in mid-cycle corpus luteum by PGF_{2α} appears to be mediated by PKC in sheep (Tsai and Wiltbank, 1997). Thus, induction of PGHS-2 in bovine granulosa cells was mediated solely by the cAMP–PKA pathway, but in granulosa-derived large luteal cells of mid-cycle ovine corpus luteum PGHS-2 expression was PKC or Ca²⁺-dependent (Tsai *et al.*, 1996; Tsai and Wiltbank, 1997). Although these results were obtained in two different species (cows and sheep), they provide a clear rationale for investigating changes in the regulation of PGHS-2 during differentiation of granulosa cells into luteal cells.

The aim of the present study was to characterize the key transitions that convert PGF_{2α}-resistant granulosa cells into PGF_{2α}-sensitive granulosa cells after long-term (10 day) culture. The hypotheses tested were: (i) LH and hCG, acting through the cAMP–PKA pathway, stimulate both PGF_{2α} receptor and PGHS-2 mRNA expression in freshly isolated granulosa cells; and (ii) as granulosa cells differentiate into cells that are similar to large luteal cells, PGF_{2α} treatment becomes inhibitory to PGF_{2α} receptor mRNA expression and eventually becomes stimulatory to PGHS-2 mRNA expression.

Materials and Methods

Chemicals and reagents

All chemicals used in this study, unless otherwise specified, were purchased from Sigma (St Louis, MO). T7 RNA polymerase and restriction enzymes were from Promega (Madison, WI). PCR2.1™ cloning system was obtained from Invitrogen (Carlsbad, CA). SuperScript™II RNase H minus M-MLV reverse transcriptase, fetal bovine serum, Taq DNA polymerase and 1 kb DNA ladders were obtained from GIBCO/BRL (Gaithersburg, MD). Magnetight™ Oligo(dT) particles were obtained from Novagen (Madison,

WI). PGF_{2α} was purchased from Cayman Co. (Ann Arbor, MI).

Isolation and culture of bovine granulosa cells

Paired bovine ovaries were collected from an abattoir and transferred to the laboratory on ice. Follicles were selected according to the following criteria: (i) pairs of ovaries had either regressing corpora lutea (preovulatory follicle) or newly ovulated corpora lutea (dominant follicle of first follicular wave); and (ii) follicular diameter was between 10 and 20 mm. Follicles that met both criteria were excised and the ovarian stroma was removed using sterile surgical tools. Follicles were washed three times with sterile PBS and the follicular fluid was aspirated using a 1 cm³ syringe with a 25 gauge needle. Concentrations of oestradiol in the follicular fluid were determined immediately using an ELISA procedure. This procedure is designed for quick analysis of high concentrations of oestradiol in follicular fluid. Briefly, primary antibody (sheep anti-oestradiol polyclonal antibody, from The Animal Reproduction and Biotechnology Laboratory, Colorado State University, Fort Collins, CO) was added to a 96-well plate pre-coated with rabbit-anti-sheep antibodies (Calbiochem, San Diego, CA) and incubated for 15 min at 37°C. After washing off excess primary antibody, 50 µl of the sample and 50 µl horseradish peroxidase (HRP)-conjugated oestradiol (made in our laboratory) were added to each well to compete for the primary antibody for 30 min at 37°C. The plate was washed four times with washing buffer (20 mmol l⁻¹ MOPS and 0.05% (v/v) Tween 20, pH 7.2). Substrate solution (125 µl; 50 mmol sodium acetate l⁻¹, pH 4.4, 0.5 mol H₂O₂ l⁻¹, and 20 mg 3,3',5,5'-tetramethyl benzidine ml⁻¹) was added to each well and incubated at 37°C for 10 min with shaking. Colour development was terminated by adding 50 µl stop solution (0.5 mol H₂SO₄ l⁻¹) to each well and absorbance was determined at 450 nm in an enzymeimmunoassay plate reader. The sensitivity (80% bound) of the oestradiol assay was 0.08 ng ml⁻¹, and the intra- and interassay coefficients of variation were 7.0 and 9.6%, respectively. Follicles with follicular fluid oestradiol concentrations ≥ 100 ng ml⁻¹ were considered as potential ovulatory follicles (Bodensteiner *et al.*, 1996). The follicles were cut in half and immersed in M199 containing 10 µg DNase I ml⁻¹ and 50 U heparin ml⁻¹. Granulosa cells were collected by gently scraping the internal surface of the follicular wall with a sterile rubber policeman. Cells were washed three times with 0.1% (w/v) BSA in M199 and counted using a haemocytometer. Cell viability was determined using 0.04% (w/v) trypan blue dye and 2 × 10⁵ viable cells per well were plated in M199 containing 100 U penicillin ml⁻¹, 100 µg streptomycin sulphate ml⁻¹, 0.1% (w/v) BSA, 1 µg insulin ml⁻¹, 100 µg bovine high density lipoprotein (HDL) ml⁻¹ and 10% fetal bovine serum (FBS) in 24-well plates at 39°C with 5% CO₂ in a humidified incubator. After overnight incubation, cells were washed three times with M199 and 0.1% (w/v) BSA to remove

unattached cells and cultured in 1 ml serum-free M199 supplemented with 100 U penicillin ml^{-1} , 100 μg streptomycin sulphate ml^{-1} , 0.1% (w/v) BSA, 1 μg insulin ml^{-1} and 100 μg HDL ml^{-1} .

In Expt 1 ($n = 5$ batches of cells), cells were cultured for 24 h with control medium, hCG (10 iu), forskolin (50 $\mu\text{mol l}^{-1}$) or phorbol didecanoate (PDD; 10 nmol l^{-1}), in the presence or absence of H89 (a selective PKA inhibitor, 1 $\mu\text{mol l}^{-1}$) or calphostin C (a selective PKC inhibitor, 0.5 $\mu\text{mol l}^{-1}$). In subsequent studies, forskolin was selected on the basis of its marked effect on induction of $\text{PGF}_{2\alpha}$ receptor mRNA and PGHS-2 mRNA and to eliminate problems due to differences in LH receptor expression. In Expt 2, a dose-response study was performed in which cells were treated with different concentrations of forskolin (0.01–10.0 $\mu\text{mol l}^{-1}$) for 24 h after overnight plating and washing ($n = 4$ batches of cells). On the basis of results obtained in this experiment, forskolin at a concentration of 10 $\mu\text{mol l}^{-1}$ was chosen for the next two experiments. In Expt 3 ($n = 3$ batches of cells), granulosa cells (prepared as above) were cultured for 1, 4, 7 or 10 days with or without 10 $\mu\text{mol forskolin l}^{-1}$. Culture media were changed each day and progesterone quantification by competitive ELISA was performed as described by Rasmussen *et al.* (1996) and Tsai and Wiltbank (1998). The assay sensitivity (80% point) was 0.16 ng ml^{-1} and the mean intra- and interassay coefficients of variation were 8 and 14%, respectively. In Expt 4 ($n = 3$ batches of cells), granulosa cells were treated with 10 $\mu\text{mol forskolin l}^{-1}$ for 1, 4, 7 or 10 days, control medium or 100 nmol $\text{PGF}_{2\alpha} \text{l}^{-1}$ was added and the culture was continued for 24 h.

RNA isolation

Poly(A⁺) RNA was isolated directly from cultured cells using Magnetight™ Oligo(dT) particles according to the manufacturer's protocol with modifications (Tsai *et al.*, 1998; Tsai and Wiltbank, 1998). Briefly, 100 μl homogenization buffer (4 mol guanidinium isothiocyanate l^{-1} , 10 mmol Tris-HCl buffer l^{-1} , pH 8.0, 0.5% (w/v) SDS and 1% (w/v) dithiothreitol) was added to each culture well to lyse granulosa cells. Chromosomal DNA was sheared by passing the homogenized solution through a 25 gauge needle 10–12 times. The cell lysate was transferred to a microcentrifuge tube and the binding buffer (200 μl ; 0.5 mol NaCl l^{-1} , 10 mmol Tris-HCl l^{-1} , pH 8.0 and 1 mmol EDTA l^{-1}) was added to the solution and mixed thoroughly. Cell debris was pelleted by centrifuging the whole lysate at 16 000 g for 5 min at 4°C and the supernatant was transferred carefully to a new microcentrifuge tube. Fifty microlitres of Magnetight™ Oligo(dT) particles were added and allowed to hybridize with mRNA at room temperature for 5 min. Magnetight™ Oligo(dT) particles were captured with a magnet stand. The supernatant was removed for determination of DNA content by Hoechst 33258 fluorescent dye and a fluorometer (Labarca and Paigen, 1980). The beads were washed five times with 500 μl

washing buffer (0.15 mol NaCl l^{-1} , 10 mmol Tris-HCl l^{-1} , pH 8.0 and 1 mmol EDTA l^{-1}). After a final wash, 15 μl elution buffer (2 mmol EDTA l^{-1}) was added to elute mRNA from the Magnetight™ Oligo(dT) particles at 65°C for 3–5 min. The mRNA was stored in aliquots at –80°C until used.

Quantitative competitive RT-PCR with a standard curve

Native and competitor RNAs for $\text{PGF}_{2\alpha}$ receptor and PGHS-2 had been constructed and used in other studies (Tsai and Wiltbank, 1996, 1997, 1998; Tsai *et al.*, 1996, 1998). Briefly, a standard curve was produced with different amounts of standard RNA co-amplified with one constant amount of competitor RNA (1 amol; Fig. 1). The number of specific mRNA transcripts in the sample was determined by RT-PCR amplification of the sample with the same amount of competitor RNA and by direct comparison of the ratio of products to the standard curve. This value was converted into molecules per cell on the basis of the DNA content in the sample. Thirty cycles of PCR (30 s at 95°C, 30 s at 57°C and 30 s at 72°C) were performed in a programmable thermocycler (PTC-100; MJ Research, Watertown, MA). The PCR was followed by a final incubation at 72°C for 5 min and the products were separated on a 5% (w/v) polyacrylamide gel in 1 × TBE (45 mmol Tris borate l^{-1} , 1 mmol EDTA l^{-1} , pH 8.0) buffer. The gel was stained with ethidium bromide and visualized under UV-transillumination. The gel image was captured with a camera connected to a Macintosh computer and analysed using Collage™ software (Fotodyne, Hartland, WI).

Statistical analysis

Data were transformed logarithmically for statistical analysis because of variation among samples. ANOVA was used to test for differences among means in all studies. When one-way ANOVA indicated a significant difference, Duncan's multiple-range test was used to compare means in each analysis. All results are shown as mean $\pm$ SEM and $P < 0.05$ was selected as the statistically significant value.

Results

Morphology of in vitro luteinized granulosa cells

Granulosa cells were attached after 16–18 h of incubation in M199 supplemented with 10% FBS. The cells flattened and grew as a monolayer during day 2 of culture in serum-free medium in the presence of forskolin or hCG (Fig. 2a). Forskolin-treated granulosa cells gradually migrated towards each other and finally formed aggregates after day 6 of culture (Fig. 2b). Cells in these aggregates had lipid droplets in the cytoplasm.

Effect of hCG and forskolin on expression of $\text{PGF}_{2\alpha}$ receptor mRNA and PGHS-2 mRNA

Granulosa cells isolated from potential ovulatory follicles did not express $\text{PGF}_{2\alpha}$ receptor or PGHS-2 (data not

Fig. 1. Schematic representation of (a) the strategy for generating native and competitor DNA standards for standard curve quantitative, competitive RT-PCR and (b,c) production of the standard curve for quantification of mRNA using prostaglandin G/H synthase (PGHS-2) as an example. (a) A native DNA fragment of 484 bp was amplified by RT-PCR using forward (P1) and reverse (P2) primers and was cloned into pCRTMII vector. An internal region of 106 bp within the native DNA fragment was deleted by *AluI* to generate the competitor DNA. (b) The ethidium bromide-stained PCR products were visualized on an UV box and the intensity of each band was quantified. M: 1 kb DNA molecular weight marker; NC: negative control omitting reverse transcriptase. (c) The ratios of native to competitor DNA were transformed logarithmically and plotted against the log of the initial amounts of native RNA added to construct the standard curve.

shown). After overnight culture in 10% FBS followed by 24 h culture in serum-free medium, steady-state concentrations of mRNA for PGF_{2α} receptor were minimal (Fig. 3a). Expression of PGF_{2α} receptor mRNA was induced by both hCG and forskolin but not by PDD (Fig. 3a). A selective PKA inhibitor, H89, decreased both hCG- and forskolin-induced expression of PGF_{2α} receptor mRNA, whereas a PKC inhibitor, calphostin C, only partially inhibited the effect of hCG (Fig. 3a). Substantial amounts of mRNA encoding PGHS-2 were observed after overnight culture in 10% FBS followed by culture in serum-free medium for 24 h (Fig. 3b). Both forskolin and hCG stimulated PGHS-2 mRNA expression, and the induction was inhibited by H89 but not

Fig. 2. Photomicrograph of bovine granulosa cells. Granulosa cells were cultured overnight in M199 + 10% fetal bovine serum (FBS) and then transferred to serum-free medium supplemented with 0.1% (w/v) BSA, 1 μg insulin ml⁻¹, 100 μg bovine high density lipoprotein (HDL) ml⁻¹ and 10 μmol forskolin l⁻¹ for (a) 2 or (b,c) 7 days. Note the lipid droplets accumulated within the cytosol of the cell (c; arrowhead). The image in (a) was obtained by using differential interference contrast microscopy and images in parts (b) and (c) were obtained under brightfield microscopy. Scale bars represent 20 μm.

by calphostin C (Fig. 3b). Treatment of granulosa cells with PDD had no effect on expression of PGHS-2 or PGF_{2α} receptor mRNA.

In Expt 2, steady-state concentrations of mRNA encoding PGF_{2α} receptor were not detected in granulosa cells treated with 0.0 or 0.01 μmol forskolin l⁻¹. The addition of 0.1, 1.0 and 10.0 μmol forskolin l⁻¹ showed a dose-dependent increase in PGF_{2α} receptor mRNA (Fig. 4). Basal concentrations of PGHS-2 mRNA (ten copies per cell) were

Fig. 3. Effects of selective activators and inhibitors of protein kinase A (PKA) (forskolin and H89, ▨) and protein kinase C (PKC) (phorbol didecanoate (PDD) and calphostin C, ■) on steady-state concentrations of (a) $\text{PGF}_{2\alpha}$ receptor mRNA and (b) prostaglandin G/H synthase (PGHS-2) mRNA in bovine granulosa cells. □, control. The inhibitors were added to the cells for 30 min before the addition of the activators. Asterisks indicate significant differences ($P < 0.05$) compared with controls.

observed in granulosa cells cultured with 0.0 or 0.01 $\mu\text{mol l}^{-1}$ forskolin. A dose-dependent increase in PGHS-2 mRNA expression was also evident in granulosa cells treated with forskolin (0.1–10.0 $\mu\text{mol l}^{-1}$) for 24 h.

In Expt 3, very little, if any, $\text{PGF}_{2\alpha}$ receptor mRNA was detected in cells cultured without forskolin for 1, 4, 7 or 10 days. Administration of 10 $\mu\text{mol l}^{-1}$ forskolin increased $\text{PGF}_{2\alpha}$ receptor mRNA > tenfold on day 1 of culture (Fig. 5). Further increases in $\text{PGF}_{2\alpha}$ receptor mRNA were observed after day 4 of culture and up to a > 1000-fold increase after day 7 of culture (Fig. 5). In the presence of forskolin, granulosa cells expressed greater amounts of PGHS-2 mRNA compared with the control groups (Fig. 5). The number of PGHS-2 transcripts in the forskolin-treated group decreased gradually with time in culture (2615 ± 1189

Fig. 4. Effect of forskolin on steady-state concentrations of mRNA encoding for $\text{PGF}_{2\alpha}$ receptor (□) and prostaglandin G/H synthase (PGHS-2) (■) in bovine granulosa cells. Freshly isolated granulosa cells were cultured overnight in M199 plus 10% fetal bovine serum and then transferred to serum-free medium and treated with different concentrations (0, 0.01, 0.10, 1.00, or 10.00 $\mu\text{mol l}^{-1}$) of forskolin for 24 h. Asterisks indicate differences ($P < 0.05$) compared with controls.

copies per cell in day 1 versus 462 ± 281 copies per cell in day 10; $P < 0.05$). Progesterone production by granulosa cells was increased > tenfold by treatment with forskolin (control versus forskolin: 19 ± 2 ng ml^{-1} versus 72 ± 7 ng ml^{-1} , 41 ± 7 ng ml^{-1} versus 272 ± 14 ng ml^{-1} , 64 ± 17 ng ml^{-1} versus 353 ± 27 ng ml^{-1} , and 31 ± 2 ng ml^{-1} versus 486 ± 112 ng ml^{-1} for days 1, 4, 7 and 10, respectively).

Effect of $\text{PGF}_{2\alpha}$ on expression of $\text{PGF}_{2\alpha}$ receptor mRNA and PGHS-2 mRNA at different stages of cultured granulosa cells

In Expt 4, the time course for induction of $\text{PGF}_{2\alpha}$ receptor mRNA and PGHS-2 mRNA was similar to that in Expt 3: maximum induction was observed at day 7 of culture for $\text{PGF}_{2\alpha}$ receptor mRNA and at day 1 for PGHS-2 mRNA (data not shown). Treatment of forskolin-treated granulosa cells with $\text{PGF}_{2\alpha}$ decreased $\text{PGF}_{2\alpha}$ receptor mRNA on days 4, 7 and 10 of culture but not on day 1 of culture (Fig. 6). Treatment of forskolin-stimulated granulosa cells with $\text{PGF}_{2\alpha}$ did not change expression of PGHS-2 mRNA on day 1 or day 4 of culture. Treatment with $\text{PGF}_{2\alpha}$ significantly increased expression of PGHS-2 mRNA on day 7 and day 10 of culture (Fig. 6).

Steady-state concentrations of mRNA encoding for the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase, did not change in response to forskolin or $\text{PGF}_{2\alpha}$ at any time point examined (data not shown).

Discussion

The first aim of this study was to analyse the regulation of mRNA for the $\text{PGF}_{2\alpha}$ receptor and PGHS-2 in granulosa cells from preovulatory follicles. Expression of $\text{PGF}_{2\alpha}$

Fig. 5. Time course for the effect of forskolin (■, $\mu\text{mol l}^{-1}$) on steady-state concentrations of mRNA for (a) $\text{PGF}_{2\alpha}$ receptor and (b) prostaglandin G/H synthase (PGHS-2) in bovine granulosa cells. □: control. Granulosa cells were cultured with or without forskolin for 1, 4, 7 or 10 days. Within (a) and (b), values with different letters are significantly different ($P < 0.05$).

receptor mRNA and PGHS-2 mRNA could be induced by either hCG or forskolin, a pharmacological stimulator of cAMP production. The effect of forskolin was dose-dependent: 10 $\mu\text{mol l}^{-1}$ induced the highest expression. In addition, the effect was inhibited by the PKA inhibitor H89, which indicates the specific involvement of PKA. Calphostin C also resulted in a small but significant inhibition of hCG-induced expression of $\text{PGF}_{2\alpha}$ receptor mRNA, implicating PKC in the action of hCG. However, the lack of induced expression of $\text{PGF}_{2\alpha}$ receptor mRNA by a specific PKC activator, PDD, is not consistent with PKC activation. This discrepancy may be due to a lack of calphostin C specificity for PKC, random error or a complex interaction between pathways in the induction of $\text{PGF}_{2\alpha}$ receptor mRNA by hCG. The major intracellular pathway inducing $\text{PGF}_{2\alpha}$ receptor mRNA and PGHS-2 mRNA is clearly the cAMP-PKA pathway and the involvement of other intracellular pathways is not strongly implicated.

These findings from *in vitro* studies are consistent with

Fig. 6. Effects of $\text{PGF}_{2\alpha}$ on steady-state concentrations of mRNA encoding $\text{PGF}_{2\alpha}$ receptor (□) and prostaglandin G/H synthase (PGHS-2) (■) in forskolin-primed bovine granulosa cells. The steady-state concentrations of mRNA in forskolin-treated groups were expressed as 100%. Asterisks indicate ($P < 0.05$) significant differences compared with forskolin-treated control.

results *in vivo*, which showed marked induction of PGHS-2 mRNA at 24 h after the LH surge and of $\text{PGF}_{2\alpha}$ receptor mRNA at 48 h after the LH surge (Tsai *et al.*, 1996). Granulosa cells appear to differentiate into large luteal cells that, at least in sheep, contain most of the luteal $\text{PGF}_{2\alpha}$ receptors (Fitz *et al.*, 1982; Juengel *et al.*, 1996). The marked induction of $\text{PGF}_{2\alpha}$ receptor in luteinized granulosa cells, the increased progesterone production and the morphology of luteinized granulosa cells were all consistent with granulosa cells differentiating into large luteal cells in a cAMP-mediated response to the LH surge. This finding is in agreement with a report by Mamluk *et al.* (1998) in which granulosa cells luteinized *in vitro* expressed greater amounts of $\text{PGF}_{2\alpha}$ receptor in response to increased concentrations of cAMP. Similarly, a study by Ristimäki *et al.* (1997) on human granulosa lutein cells revealed that hCG stimulated expression of $\text{PGF}_{2\alpha}$ receptor mRNA in a concentration-dependent manner. In contrast, Vaananen *et al.* (1998) reported that hCG inhibited $\text{PGF}_{2\alpha}$ receptor expression in short term (1 day) cultures of human granulosa lutein cells. In the present study, Expt 3 demonstrated that forskolin continues to be stimulatory to $\text{PGF}_{2\alpha}$ receptor expression during long-term culture of bovine granulosa cells and maximum stimulation occurred at day 7 of culture. A study *in vivo* by Tsai *et al.* (1996) reported maximum $\text{PGF}_{2\alpha}$ receptor expression at day 2 after ovulation (approximately 3 days after the LH surge). The later occurrence of maximum $\text{PGF}_{2\alpha}$ receptor expression under conditions *in vitro* may be due to multiple culture factors that were absent *in vivo* including the continuous presence of forskolin during the studies *in vitro*. Expression of PGHS-2 mRNA was at a maximum 24 h after forskolin treatment and decreased at days 4, 7 and 10 despite the continual presence of forskolin. Thus, although cAMP

stimulates the expression of both PGF_{2α} receptor mRNA and PGHS-2 mRNA in granulosa cells there are different temporal mechanisms that regulate these responses.

Sirois and Dore (1997) suggested that production of prostaglandins in the preovulatory follicle is an important signal that sets the timing of ovulation in mammals. Although both PGHS-1 and PGHS-2 regulate the rate-limiting step in prostaglandin biosynthesis, expression of PGHS-2 is the critical regulator of follicular prostaglandin production (Sirois *et al.*, 1992; Sirois, 1994; Tsai *et al.*, 1996; Sirois and Dore, 1997). Bovine granulosa cells do not express PGHS-2 mRNA and protein *in vivo* until 18–24 h after the LH surge (Sirois, 1994; Tsai *et al.*, 1996). The subsequent production of prostaglandins, particularly PGE₂, may be involved in dissolution of the follicular basement membrane and apoptosis of the overlying surface epithelial cells (Ackerman and Murdoch, 1993). At present, the physiological importance of induction of PGF_{2α} receptor in the early corpus luteum is unclear. The early corpus luteum does not regress in response to a single injection of PGF_{2α} despite the presence of normal numbers of PGF_{2α} receptors (Tsai and Wiltbank, 1998). It is possible that PGF_{2α} has early effects on the development of structural or functional aspects of the corpus luteum.

The second aim of the present study was to test the hypothesis that luteinized granulosa cells acquire responsiveness to PGF_{2α} in a manner that is similar to acquisition of PGF_{2α} responsiveness *in vivo*. The actions of PGF_{2α} that result in luteal regression are mediated by binding to the PGF_{2α} receptor, as demonstrated by lack of both luteal regression and parturition in PGF_{2α} receptor knockout mice (Sugimoto *et al.*, 1997). Thus, the initial expression of PGF_{2α} receptor mRNA and protein would be expected to occur before detection of PGF_{2α} responsiveness. Other studies demonstrated that PGF_{2α} treatment decreased steady-state concentrations of PGF_{2α} receptor mRNA in day 4 or day 11 bovine corpus luteum (Tsai and Wiltbank, 1998), in mid-cycle ovine corpus luteum (Tsai and Wiltbank, 1997), and in bovine granulosa luteal cells luteinized *in vitro* (Mamluk *et al.*, 1998). Similarly, in the present study, PGF_{2α} inhibited forskolin-stimulated PGF_{2α} receptor mRNA at days 4, 7 and 10 after the start of luteinization treatment. In bovine granulosa cells cultured without forskolin (S.-J. Tsai and C. Wiltbank, unpublished) or in those that received treatment with forskolin for 1 day, PGF_{2α} had no effect, probably due to a lack of PGF_{2α} receptors. The physiological importance of inhibition of PGF_{2α} receptor mRNA by PGF_{2α} is unclear, but may be similar to the downregulatory response found for other hormones, such as LH (Peng *et al.*, 1991; Meduri *et al.*, 1996).

In contrast to PGF_{2α} receptor mRNA, treatment with PGF_{2α} increased PGHS-2 mRNA content. PGF_{2α}-induced stimulation of PGHS-2 mRNA and protein has been reported in cultured ovine large luteal cells (Tsai and Wiltbank, 1997) and after *in vivo* treatment of sheep (Tsai and Wiltbank, 1997), pigs (Diaz *et al.*, 2000) or cattle (Tsai and Wiltbank, 1998). Of particular importance is the

finding that PGF_{2α} stimulated PGHS-2 mRNA in day 11 but not day 4 bovine corpus luteum (Tsai and Wiltbank, 1998). Similarly, in the present study, bovine granulosa cells cultured with forskolin showed no effect of PGF_{2α} on PGHS-2 mRNA after 1 or 4 day of culture; however, PGF_{2α} markedly stimulated the expression of PGHS-2 mRNA after 7 and 10 days of culture with forskolin. This finding is similar to the timing for acquisition of luteolytic capacity in cattle. Treatment with PGF_{2α} causes luteolysis in cattle after day 7 of the oestrous cycle (7 days after the LH surge), but not before day 7 (Momont and Seguin, 1984). Thus, as found *in vivo*, luteinization of granulosa cells induces expression of PGF_{2α} receptor and subsequent responsiveness to PGF_{2α}, as demonstrated by PGF_{2α}-induced inhibition of PGF_{2α} receptor mRNA at day 4 of culture. However, acquisition of responsiveness of PGHS-2 mRNA to PGF_{2α} requires additional time and possibly further differentiation of granulosa cells. Considering the key role of PGHS-2 in numerous cellular responses, it is of particular interest to understand the molecular mechanisms by which granulosa cells, after 7 days of culture with forskolin, acquire the ability for PGHS-2 mRNA induction by PGF_{2α}. In non-luteinized granulosa cells, cAMP–PKA is the key stimulatory pathway for PGHS-2 mRNA, whereas it seems likely that, similar to large luteal cells (Tsai and Wiltbank, 1997), the free intracellular calcium–PKC effector system is critical for PGHS-2 expression in luteinized granulosa cells. The molecular mechanisms involved in this switch in responsiveness require further study.

Although there is clear evidence regarding the primary role of uterine PGF_{2α} in the initiation of luteolysis in ruminants, the physiological importance of intraluteal production of PGF_{2α} has not been clearly delineated (Shemesh and Hansel, 1975; Guthrie *et al.*, 1978; Guthrie and Rexroad, 1980; Milvae and Hansel, 1983; Rodgers *et al.*, 1988; Nothnick and Pate, 1990). Luteal slices collected from sheep or pigs that had been treated *in vivo* with cloprostenol (PGF_{2α} analogue) produced much more PGF_{2α} than did luteal slices collected from control animals (Rexroad and Guthrie, 1979; Guthrie and Rexroad, 1980). The PGF_{2α}-induced increase in luteal PGF_{2α} production appears to be due to induction of PGHS-2 by PKC in large luteal cells (Tsai and Wiltbank, 1997, 1998). Thus, there is a positive feedback loop within large luteal cells such that a small amount of PGF_{2α} causes production of high local concentrations of PGF_{2α} within luteal tissue (Tsai and Wiltbank, 1997). It is not yet clear whether intraluteal production of PGF_{2α} is essential for luteolysis; however, it appears that this autoamplification cascade, as evidenced by PGHS-2 expression, is not induced by PGF_{2α} in granulosa cells during the early stages of luteinization despite the presence of PGF_{2α} receptor. Similarly, Levy *et al.* (2000) found that induction of endothelin 1 and its type A receptor by PGF_{2α} occurred in slices from day 10 bovine corpus luteum (with luteolytic capacity), but not in slices from day 4 corpus luteum (without luteolytic capacity).

In conclusion, production of prostaglandins in the ovary is closely associated with the two key ovarian transitions: ovulation and luteolysis. The PGHS-2 protein controls the production of prostaglandins in granulosa cells or granulosa cell-derived large luteal cells, but apparently by distinct cellular effector systems. In preovulatory granulosa cells, PGHS-2 and PGF_{2α} receptor mRNAs were increased in response to cAMP-PKA. Induction of PGF_{2α} receptor appears to be sufficient for PGF_{2α}-induced inhibition of PGF_{2α} receptor mRNA but not for PGF_{2α}-induced stimulation of PGHS-2 mRNA. The transition to PGF_{2α} responsiveness of PGHS-2 gene expression occurs about 7 days after forskolin treatment and may involve specific transcription factors that allow PKC induction of PGHS-2. This may be part of the critical transition that produces a corpus luteum that is sensitive to PGF_{2α}-induced luteolysis.

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