Differential effects of prostaglandin F2α 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 PGF2α receptors were investigated in bovine granulosa cells at various stages of differentiation. Firstly, the induction of PGF2α 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 µmol l⁻¹) for 24 h. Forskolin increased steady-state concentrations of mRNA for PGHS-2 (> 20-fold) and PGF2α receptor (> 1000-fold) in a dose-dependent fashion. Use of selective protein kinase A inhibitor (H89) reduced both hCG- and forskolin-induced expression of PGF2α receptor mRNA and PGHS-2 mRNA. The hypothesis that luteinized granulosa cells would acquire PGF2α responsiveness similar to responses to PGF2α observed *in vivo* was also evaluated. Treatment with PGF2α (100 nmol l⁻¹) reduced forskolin-induced expression of PGF2α receptor mRNA on days 4, 7 and 10, but not on day 1 of culture (n = 3). Treatment with PGF2α did not change forskolin-induced expression of PGHS-2 mRNA on or before day 4 of culture. In contrast, PGF2α significantly increased PGHS-2 mRNA expression in granulosa cells primed with forskolin for 7 or 10 days. In conclusion, expression of PGHS-2 and PGF2α receptor mRNA is protein kinase A-dependent in preovulatory bovine granulosa cells. Granulosa cells become PGF2α-responsive soon after expression of PGF2α receptor, whereas further differentiation is required before PGF2α induces PGHS-2 mRNA upregulation. These results demonstrate that at least two key transitions are required in PGF2α-induced luteal regression in the mid-cycle corpus luteum.

The action of PGF2α is mediated through binding to its plasma membrane receptor, the G protein-coupled PGF2α receptor. Binding of PGF2α by PGF2α receptor activates phospholipase C, increases free intracellular calcium concentrations and activates protein kinase C (PKC) (Wiltbank et al., 1991). In bovine granulosa cells, PGF2α 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 PGF2α receptor can be induced by forskolin in a similar time course (Tsai et al., 1996). Treatment *in vivo* with PGF2α decreased expression of PGF2α 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, PGF2α decreased expression of PGF2α receptor in cultured human granulosa lutein cells (Ristimaki et al., 1997). In contrast, PGF2α receptor expression was upregulated by PGF2α in mouse corpus luteum (Sugatani et al., 1996). It is unclear whether the different effects of PGF2α on the expression of PGF2α receptor are due to species differences or to the different stages at which the cells were treated.

One rate-limiting step in PGF2α biosynthesis is the conversion of arachidonic acid to PGH2 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$_{2\alpha}$ markedly increased luteal PGHS-2 mRNA (Tsai and Wiltbank, 1997, 1998). Induction of PGHS-2 in mid-cycle corpus luteum by PGF$_{2\alpha}$ 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 Ca$^{2+}$-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$_{2\alpha}$-resistant granulosa cells into PGF$_{2\alpha}$-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$_{2\alpha}$ 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$_{2\alpha}$ treatment becomes inhibitory to PGF$_{2\alpha}$ 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$_{2\alpha}$ 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 cm$^3$ 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 H$_2$O$_2$ 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 H$_2$SO$_4$ 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 l$^-1$ 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 × 10$^5$ 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% CO$_2$ in a humidified incubator. After overnight incubation, cells were washed three times with M199 and 0.1% (w/v) BSA to remove...

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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 µIU), forskolin (50 µmol l−1) or phorbol didecanoate (PDD; 10 nmol l−1), in the presence or absence of H89 (a selective PKA inhibitor, 1 µmol l−1) or calphostin C (a selective PKC inhibitor, 0.5 µmol l−1). In subsequent studies, forskolin was selected on the basis of its marked effect on induction of PGF2α 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 µmol 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 µmol 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 µmol 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). 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, 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).

**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., 1996, 1997, 1998; Tsai et al., 1996, 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 PGF2α 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 ± 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 PGF2α receptor mRNA and PGHS-2 mRNA**

Granulosa cells isolated from potential ovulatory follicles did not express PGF2α receptor or PGHS-2 (data not
shown). After overnight culture in 10% FBS followed by 24 h culture in serum-free medium, steady-state concentrations of mRNA for PGF$_{2\alpha}$ receptor were minimal (Fig. 3a). Expression of PGF$_{2\alpha}$ 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$_{2\alpha}$ 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 by calphostin C (Fig. 3b). Treatment of granulosa cells with PDD had no effect on expression of PGHS-2 or PGF$_{2\alpha}$ receptor mRNA.

In Expt 2, steady-state concentrations of mRNA encoding PGF$_{2\alpha}$ receptor were not detected in granulosa cells treated with 0.0 or 0.01 µmol forskolin 1$^{-1}$. The addition of 0.1, 1.0 and 10.0 µmol forskolin 1$^{-1}$ showed a dose-dependent increase in PGF$_{2\alpha}$ receptor mRNA (Fig. 4). Basal concentrations of PGHS-2 mRNA (ten copies per cell) were
observed in granulosa cells cultured with 0.0 or 0.01 μmol forskolin l⁻¹. A dose-dependent increase in PGHS-2 mRNA expression was also evident in granulosa cells treated with forskolin (0.1–10.0 μmol l⁻¹) for 24 h.

In Expt 3, very little, if any, PGF2α receptor mRNA was detected in cells cultured without forskolin for 1, 4, 7 or 10 days. Administration of 10 μmol forskolin l⁻¹ increased PGF2α receptor mRNA > tenfold on day 1 of culture (Fig. 5). Further increases in PGF2α 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 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 PGF2α on expression of PGF2α receptor mRNA and PGHS-2 mRNA at different stages of cultured granulosa cells**

In Expt 4, the time course for induction of PGF2α receptor mRNA and PGHS-2 mRNA was similar to that in Expt 3: maximum induction was observed at day 7 of culture for PGF2α receptor mRNA and at day 1 for PGHS-2 mRNA (data not shown). Treatment of forskolin-stimulated granulosa cells with PGF2α decreased PGF2α 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 PGF2α did not change expression of PGHS-2 mRNA on day 1 or day 4 of culture. Treatment with PGF2α 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 PGF2α at any time point examined (data not shown).

**Discussion**

The first aim of this study was to analyse the regulation of mRNA for the PGF2α receptor and PGHS-2 in granulosa cells from preovulatory follicles. Expression of PGF2α
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 μmol forskolin 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 PGF₂α receptor mRNA, implicating PKC in the action of hCG. However, the lack of induced expression of PGF₂α 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 PGF₂α receptor mRNA by hCG. The major intracellular pathway inducing PGF₂α 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 results in vivo, which showed marked induction of PGHS-2 mRNA at 24 h after the LH surge and of PGF₂α 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 PGF₂α receptors (Fitz et al., 1982; Juengel et al., 1996). The marked induction of PGF₂α 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 PGF₂α receptor in response to increased concentrations of cAMP. Similarly, a study by Ristimaki et al. (1997) on human granulosa lutein cells revealed that hCG stimulated expression of PGF₂α receptor mRNA in a concentration-dependent manner. In contrast, Vaananen et al. (1998) reported that hCG inhibited PGF₂α 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 PGF₂α 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 PGF₂α receptor expression at day 2 after ovulation (approximately 3 days after the LH surge). The later occurrence of maximum PGF₂α 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$_{2\alpha}$ 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 PGE$_2$, 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$_{2\alpha}$ receptor in the early corpus luteum is unclear. The early corpus luteum does not regress in response to a single injection of PGF$_{2\alpha}$ despite the presence of normal numbers of PGF$_{2\alpha}$ receptors (Tsai and Wiltbank, 1998). It is possible that PGF$_{2\alpha}$ 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$_{2\alpha}$ in a manner that is similar to acquisition of PGF$_{2\alpha}$ responsiveness in vivo. The actions of PGF$_{2\alpha}$ that result in luteal regression are mediated by binding to the PGF$_{2\alpha}$ receptor, as demonstrated by lack of both luteal regression and parturition in PGF$_{2\alpha}$ receptor knockout mice (Sugimoto et al., 1997). Thus, the initial expression of PGF$_{2\alpha}$ receptor mRNA and protein would be expected to occur before detection of PGF$_{2\alpha}$ 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$_{2\alpha}$-inhibited forskolin-stimulated PGF$_{2\alpha}$ 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$_{2\alpha}$ had no effect, probably due to a lack of PGF$_{2\alpha}$ receptors. The physiological importance of inhibition of PGF$_{2\alpha}$ receptor mRNA by PGF$_{2\alpha}$ 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$_{2\alpha}$ receptor mRNA, treatment with PGF$_{2\alpha}$ increased PGHS-2 mRNA content. PGF$_{2\alpha}$-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$_{2\alpha}$ 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$_{2\alpha}$ on PGHS-2 mRNA after 1 or 4 day of culture; however, PGF$_{2\alpha}$ 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$_{2\alpha}$ 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$_{2\alpha}$ receptor and subsequent responsiveness to PGF$_{2\alpha}$, as demonstrated by PGF$_{2\alpha}$-induced inhibition of PGF$_{2\alpha}$ receptor mRNA at day 4 of culture. However, acquisition of responsiveness of PGHS-2 mRNA to PGF$_{2\alpha}$ 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$_{2\alpha}$. 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$_{2\alpha}$ in the initiation of luteolysis in ruminants, the physiological importance of intraluteal production of PGF$_{2\alpha}$ 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$_{2\alpha}$ analogue) produced much more PGF$_{2\alpha}$ than did luteal slices collected from control animals (Rexroad and Guthrie, 1979; Guthrie and Rexroad, 1980). The PGF$_{2\alpha}$-induced increase in luteal PGF$_{2\alpha}$ 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$_{2\alpha}$ causes production of high local concentrations of PGF$_{2\alpha}$ within luteal tissue (Tsai and Wiltbank, 1997). It is not yet clear whether intraluteal production of PGF$_{2\alpha}$ is essential for luteolysis; however, it appears that this autoamplification cascade, as evidenced by PGHS-2 expression, is not induced by PGF$_{2\alpha}$ in granulosa cells during the early stages of luteinization despite the presence of PGF$_{2\alpha}$ receptor. Similarly, Levy et al. (2000) found that induction of endothelin 1 and its type A receptor by PGF$_{2\alpha}$ 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_{2\alpha} receptor mRNAs were increased in response to cAMP–PKA. Induction of PGF_{2\alpha} receptor appears to be sufficient for PGF_{2\alpha}-induced inhibition of PGF_{2\alpha} receptor mRNA but not for PGF_{2\alpha}-induced stimulation of PGHS-2 mRNA. The transition to PGF_{2\alpha} 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_{2\alpha}-induced luteolysis.

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