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# Intraluteal Release of Prostaglandin $F_{2\alpha}$ and $E_2$ During Corpora Lutea Development in the Cow

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**Abstract.** It is well known that prostaglandin (PG) $F_{2\alpha}$ and $PGE_2$ are actively produced by bovine early corpus luteum (CL) at higher levels than in the CL of later stages. However, there is no *in vivo* information about the local secretion of PGs within bovine developing CL so far. Thus, the objective of the present study was to determine in detail the real-time changes in $PGF_{2\alpha}$ and $PGE_2$ secretion within developing CL. A microdialysis system (MDS) was surgically implanted into the newly formed CL of 6 cows on Day 3 after GnRH administration (about 1.5 days after ovulation) to induce superovulation following FSH treatment. Both $PGF_{2\alpha}$ and $PGE_2$ release were high during the first 24 h within developing CL but decreased thereafter. These profiles were well reflected in the PG concentrations in ovarian venous plasma. Furthermore, reverse transcription polymerase chain reaction analysis using CL from other animals independent from the MDS study revealed that the level of cyclo-oxyganase-2 mRNA expression in early CL was higher than that of later luteal phases. The present results provide the first direct *in vivo* evidence that active PGs production in CL occurs up to Day 4 after estrus in the cow.
Key words: $PGF_{2\alpha}$, $PGE_2$, Developing CL, Cow, *In vivo*

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After ovulation, the new corpus luteum (CL) rapidly develops from the wall of the ruptured follicle within a few days. It is well known that basic fibroblast growth factor (bFGF) [1] and vascular endothelial growth factor (VEGF) [2] stimulate angiogenesis, which is indispensable for CL development. Besides their angiogenic activity, these growth factors stimulate prostaglandin (PG) $F_{2\alpha}$ [3, 4] and $PGE_2$ [3] synthesis from bovine early CL *in vitro*. It has been shown that both PGs are actively produced in early CL at higher levels than in other luteal stages [5, 6]. The bovine CL has

binding sites for $PGF_{2\alpha}$ [7, 8] and $PGE_2$ [9]. Furthermore, many studies have repeatedly reported that $PGF_{2\alpha}$ stimulates progesterone (P) synthesis from luteal cells and CL tissue [4, 10–14]. Likewise, $PGE_2$ is also capable of stimulating P synthesis *in vitro* [3, 14]. These findings suggest that active PGs production within developing CL supports the mechanism of P production. However, no *in vivo* information about the local secretion of PGs within bovine developing CL has yet been reported. Thus, the objective of the present study was to determine in detail the real-time changes in both PGs concentrations within the developing CL by using a microdialysis system (MDS), in conjunction with the measurement of

changes in ovarian venous plasma (OVP) ipsilateral to the CL as well as in jugular venous plasma (JVP) in the cow. Moreover, the relative changes in mRNA expression for cyclo-oxygenase (Cox-2) were analyzed by reverse transcription polymerase chain reaction (RT-PCR).

## Materials and Methods

### Animals

Six multiparous, non-lactating Brown Swiss cows were used for the *in vivo* MDS study. In order to implant multiple MDS lines in different CL in the same ovary (cow), the cows were superovulated by eight doses of ovine FSH (Ovagen; Immuno-chemical products LTD, Auckland, New Zealand), 18 mg in total. This treatment enabled us to observe local secretions in greater numbers of developing CL than a single CL formed after spontaneous ovulation. A schematic time schedule of the superovulatory treatment and MDS is shown in Fig. 1. Three days after GnRH analog (fertirelin acetate: 100 $\mu$g) injection, a laparotomy was performed, as described previously [15] to surgically implant the MDS membranes into the newly formed CL. The day of GnRH treatment was designated as Day 0. After surgery, the cows were moved to individual stanchions, where they were fed daily with corn silage and hay with free access to water. At the end of the experiment (Day 6 after the GnRH injection), the cows were ovariectomized and the ovaries were visually inspected to confirm the CL development and the location of the MDS within the CL.

### Implantation of the MDS capillaries into the early CL

The *in vivo* MDS for bovine CL was applied as described previously [15]. Basically, one or two dialysis membranes (Fresenius SPS 900 Hollow Fibers, cutoff $M_r$ = 1000 kDa, 0.2 mm in diameter, 5 mm long; Fresenius AG, St. Wendel, Germany) were implanted into early CL with a 25-gauge hypodermic needle. Both ends of the membrane were glued to a 25-cm-long piece of silicone elastomer tubing (i.d. 0.3 mm). The tubing was fixed on the surface of the early CL by Histoacryl blau (B. Braun-Dexon GmbH, Spangenberg, Germany), and the dialysis pieces with silicone tubing were connected to Teflon tubing that led to the outside of the abdomen. One end of the MDS was connected to a multiple-line peristaltic pump, and the other was connected to a multiple-line fraction collector. The MDS was continuously perfused with Ringer's solution at a flow rate of 2.5 ml/h throughout the experiments, and the fractions of the perfusates were collected at intervals of 4 h starting on Day 3 for the next 4 days.

At the time of surgery, an 18-gauge catheter (Medicut Catheter Kit; Argyle Co., Japan Sherwood, Tokyo, Japan) was inserted into the ovarian vein ipsilateral to the implanted MDS, and a jugular venous catheter was also implanted. Samples of JVP and OVP for determination of PGs and P were collected at 4-h intervals into sterile 10-ml tubes containing 200 $\mu$l of a stabilizer solution (0.3 M EDTA, 1% acid acetyl salicylic, pH 7.4). All tubes were immediately chilled in ice water for 10 min, centrifuged at 2000 × g for 10 min at 4 C, and the obtained plasma was frozen at –30 C until further analysis.
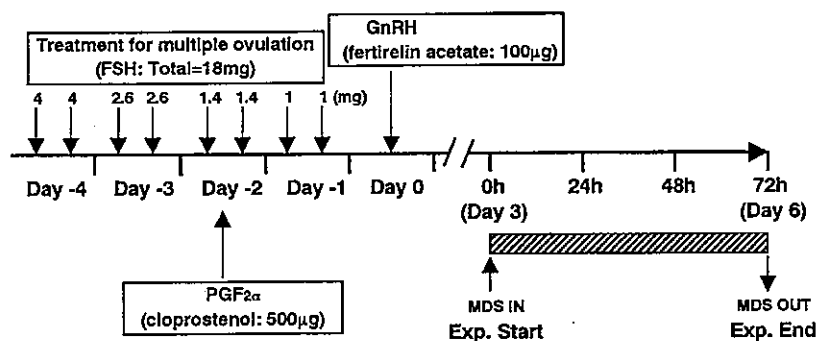


**Fig. 1.** Time schedule of the treatment for multiple ovulation and an *in vivo* MDS of bovine early CL.

## PGs extraction

The plasma samples (OVP and JVP: 2 ml) and the MDS perfusates (8 ml) were adjusted to pH 3.5 and extracted using diethyl ether as described previously [16]. The residue was dissolved in 200 $\mu$l assay buffer (40 mM PBS, 0.1% BSA, pH 7.2) for plasma samples and in 300 $\mu$l for MDS perfusates. The recovery rate of plasma $PGF_{2\alpha}$ and $PGE_2$ were validated earlier as 64% and 61%, respectively. To estimate the recovery rate in the MDS perfusate, $PGF_{2\alpha}$ and $PGE_2$ were added to the Ringer's solution (10 pg/ml), and the obtained values were 65% and 61%, respectively.

## Hormone determination

Concentrations of P were assayed as described in detail earlier [17]. The standard curve of P ranged from 0.05 to 50 ng/ml, and the $ED_{50}$ of the assay was 1.8 ng/ml. The intra- and inter-assay coefficients of variation (CVs) were on average 6.2% and 9.3%, respectively. The EIA for $PGF_{2\alpha}$ and $PGE_2$ were carried out as described previously [18]. The standard curve of $PGF_{2\alpha}$ ranged from 19.5 to 2,000 pg/ml, and the $ED_{50}$ of the assay was 355 pg/ml. The intra- and inter-assay CVs were on average 7.8% and 11.5%, respectively. The standard curve of $PGE_2$ ranged from 30 to 14,200 pg/ml, and the $ED_{50}$ of the assay was 350 pg/ml. The intra- and inter-assay CVs were on average 9.5% and 12.5%, respectively.

## CL collection for RNA extraction

The CL from Brown Swiss cows were collected at a local slaughterhouse within 10–20 minutes after slaughter. The stage of the estrous cycle was defined by macroscopic observation of the uterus and ovaries (follicles and CL). The characteristics of the ovaries that were studied included size, color, consistency, connective tissue, thickness of the endometrium, mucus, and absence of elongated early embryos. The luteal stages were classified into 4 levels: early (stage I: Days 1–4; Day 1=day of ovulation), mid (stage II: Days 5–10), late (stage III: Days 11–17) and regressed (stage IV: Days 18–20) as described by Ireland *et al.* [19]. To estimate the quantity of mRNA for Cox-2, CL were separated from the ovaries immediately after determination of the stages, frozen rapidly in liquid nitrogen, and then stored at –80 C until processed for studies of gene expression.

## RNA isolation and RT-PCR

Total RNA from bovine CL was isolated by the single-step method of Chomczynski and Sacchi [20] with Trizol reagent (Gibco BRL, Gaithersburg, MD, USA).

Two micrograms of total RNA were used to generate single-strand cDNA in a 60-$\mu$l reaction mixture as described previously [21]. PCR amplification of Cox-2 analysis consisted of one denaturing step at 94 C for 2 min, followed by 38 cycles of 94 C for 30 sec and 64 C for 45 sec. One additional extension step was carried out at 72 C for 2 min. Samples for the house-keeping gene ubiquitin were amplified for 20 cycles: a single denaturation step at 94 C for 2 min, followed by cycles of 94 C for 45 sec and 55 C for 45 sec, with the final extension step at 72 C for 2 min.

To determine the optimal quantity of reverse transcript needed for PCR and to verify that the cDNA product was dependent on the amount of transcript used, varying quantities of transcript template were used in the PCR reaction process. The primers were designed to encode the bovine sequences using the European Molecular Biology Laboratory (EMBL) database, or were used as described elsewhere, and were commercially synthesized (Amersham-Pharmacia, Freiburg, Germany). The primers were chosen using the HUSAR online programme package in Heidelberg (http://genome.dkfz-heidelberg.de). The primers were as follows: Cox-2 forward 5'-CTCTTCCTCCTGT-GCCTGAT-3' and reverse 5'-GACTCATAGAAACTGACACCCTC-3' (EMBL Ac. No. AF004944); ubiquitin forward 5'-ATGCAGATCTTTGTGAAGAC-3' and reverse 5'-CTTCTGGATGTTGTAGTC-3'. The predicted sizes of the resulting RT-PCR products were 359 base pairs (bp) for Cox-2 and 189 bp for ubiquitin respectively.

Aliquots of the PCR reaction products (5 $\mu$l) were fractionated by electrophoresis through a 1.5% agarose gel containing ethidium bromide in a constant 60-V field. The resultant band intensities were scanned by a video documentation system (Amersham-Pharmacia) and analyzed with the Image Master ID program (Amersham-Pharmacia). Each PCR product was confirmed by use of direct PCR product sequencing (TopLab, Munich, Germany). The relative signal intensitie of PCR products specific for Cox-2 were assessed after correction, based on the ubiquitin signal intensities.

**Table 1.** Comparison of P, $PGF_{2\alpha}$ and $PGE_2$ concentrations between jugular venous plasma (JVP) and ovarian venous plasma (OVP) during the early luteal phase (mean ± SEM, n=6 cows)

| Hormone | Time (h) | | |
|---|---|---|---|
| | 0-24 | 24-48 | 48-72 |
| P (ng/ml) | | | |
| JVP | $9.2 \pm 1.3^a$ | $13.7 \pm 2.3^b$ | $18.7 \pm 2.7^b$ |
| OVP | $942 \pm 137^{***a}$ | $1376 \pm 182^{***b}$ | $1425 \pm 173^{***b}$ |
| $PGF_{2\alpha}$ (pg/ml) | | | |
| JVP | $18.2 \pm 1.5$ | $12.5 \pm 1.0$ | $17.3 \pm 1.2$ |
| OVP | $43.0 \pm 4.5^{***a}$ | $19.2 \pm 1.2^{*b}$ | $21.5 \pm 1.6^b$ |
| $PGE_2$ (pg/ml) | | | |
| JVP | $15.6 \pm 3.1^a$ | $5.8 \pm 0.9^b$ | $7.9 \pm 1.0^b$ |
| OVP | $22.9 \pm 4.9^{*a}$ | $9.5 \pm 1.0^b$ | $12.7 \pm 1.2^b$ |

* $P<0.05$, *** $P<0.001$ vs. values of JVP during same period.
a,b: $P<0.05$ between different periods.

## Statistical analysis

The statistical significance of differences in mRNA expressions of Cox-2 was assessed by analysis of ANOVA, followed by the Tukey-Kramer test as a multiple comparison test. The level of significance was set at $P<0.05$. All experimental data are shown as the mean ± SEM.

The mean hormone ($PGF_{2\alpha}$ and $PGE_2$) concentrations in the MDS perfusate in the first 24-h fraction were used to calculate the individual baseline because of a large variations in the basal concentrations of each hormone released into the MDS lines implanted in the different CL ($PGF_{2\alpha}$: 5.5–77.0 pg/ml, $PGE_2$: 1.5–26.4 pg/ml). All hormone concentrations were expressed as a proportion of this individual baseline. This treatment enabled evaluation of relative changes of hormonal values between the different CL. Means were analysed by ANOVA followed by the Tukey-Kramer test as a multiple comparison test. For the figures of MDS data, all hormone concentrations in the fractions were then expressed as a percentage of this individual baseline. The absolute concentrations of each hormone during the first 24 h (baseline) of an experiment are given in the figure legends. The mean concentrations of P, $PGF_{2\alpha}$ and $PGE_2$ in OVP and JVP samples collected at different time periods were compared on the basis of each 24-h period. To compare the values of concentrations between OVP and JVP, the mean values of each 24-h period were analyzed by ANOVA followed by Student's *t*-test. The mean absolute concentrations of P and PGs were analyzed on the basis of the above 24-h period

throughout the experiment by ANOVA followed by the Tukey-Kramer test as a multiple comparison test.

## Results

The CL had developed to at least 1 cm in diameter when the cows were ovariectomized at the end of the experiment (Day 6 after GnRH injection).

### Plasma changes in P during CL development

Changes in P concentrations in OVP and JVP during CL development are shown in Table 1. Plasma P concentrations gradually increased from Day 3 to Day 6 after GnRH injection ($P<0.05$; Table 1). The P levels in OVP were about 100 times higher than those in JVP ($P<0.001$; Table 1)

### Intraluteal and plasma changes in $PGF_{2\alpha}$ and $PGE_2$ during CL development

Changes in $PGF_{2\alpha}$ and $PGE_2$ concentrations in the MDS fractions and in OVP and JVP during CL development are shown in Figs. 2 and 3. Both $PGF_{2\alpha}$ and $PGE_2$ release in the MDS fractions were high during the first 24 h of the experimental period (Days 3–4). This profile was well reflected in the plasma PGs concentrations (Figs. 2 and 3, Table 1).

### Expression of mRNA for Cox-2

Specific transcript for Cox-2 was detected in bovine CL. Each PCR product showed 100%
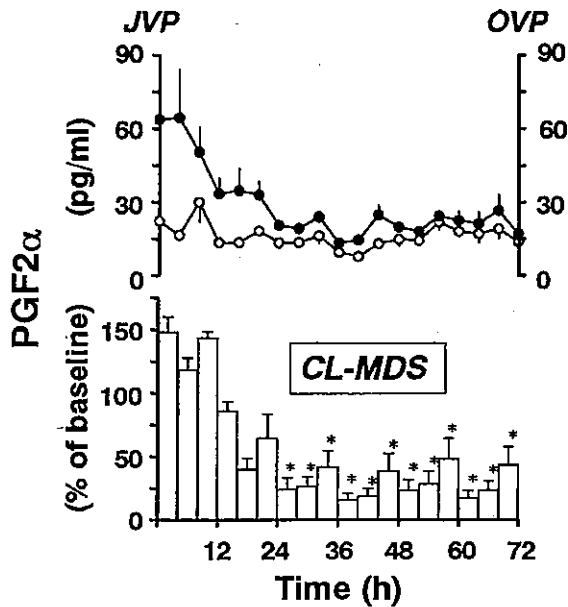
Fig. 2. Local release of $PGF_{2\alpha}$ into MDS (bar graphic: n=23 lines) and $PGF_{2\alpha}$ concentrations in jugular venous plasma (JVP) and ovarian venous plasma (OVP) (line graphic: n=6 cows) during CL development. The closed circles show OVP and the open circles show JVP. Baseline concentrations (100%) in the MDS perfusate were $6.0 \pm 0.3$ pg/ml for $PGF_{2\alpha}$. * P<0.05 vs baseline.
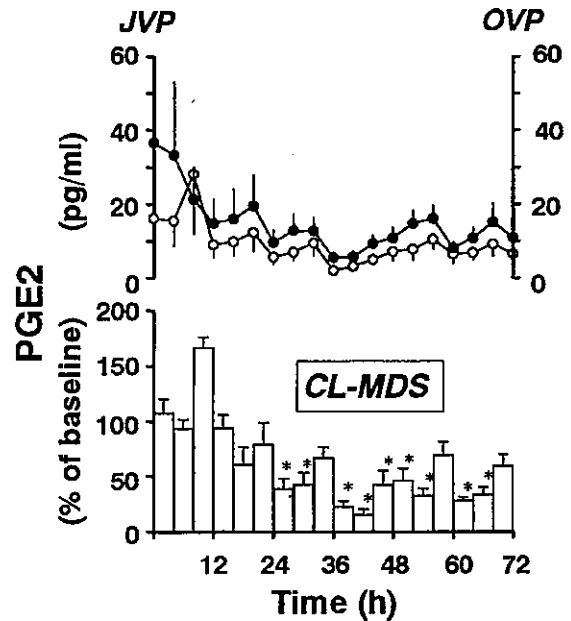
Fig. 3. Local release of $PGE_2$ into the MDS (bar graphic: n=23 lines) and $PGE_2$ concentrations in jugular venous plasma (JVP) and ovarian venous plasma (OVP) (line graphic: n=6 cows) during CL development. The closed circles show OVP and the open circles show JVP. Baseline concentrations (100%) in the MDS perfusate were $8.4 \pm 1.0$ pg/ml for $PGE_2$. * P<0.05 vs baseline.

homology to the known bovine genes after sequencing. The relative signal intensities of PCR products specific for Cox-2 were assessed after collection, based on the ubiquitin signal intensities.

The results of the densitometric analysis of Cox-2 mRNA in the CL tissue during the estrous cycle are shown in Fig. 4. The mRNA expression for Cox-2 was higher (P<0.05) at the early stage than at other luteal stages.

## Discussion

The present study provides the first *in vivo* information that active $PGF_{2\alpha}$ and $PGE_2$ release within developing CL during the early luteal phase is limited to the first 3 days after ovulation, and this profile is well reflected in the ovarian venous plasma concentrations. There is common agreement that the production of PGs by bovine early CL is higher that at other luteal phases [5, 6]. During surgery for implanting MDS, a little

bleeding was observed on the surface of the CL. Therefore, we suspected the possibility that the active release of PGs in this study may have resulted from damage to tissue during implantation of MDS. Thus, CL were collected from other animals independent from the MDS study. The level of the Cox-2 mRNA in early CL was clearly higher than at later luteal phases, indicating that active PGs production observed in the MDS study was not due to tissue damage.

We and others have shown that an acute increase of PGs in bovine mature follicles is observed during the periovulatory period [22, 23]. An acute increase of $PGF_{2\alpha}$ concentration in OVP as well as in the MDS perfusate was observed about 30 h after the LH surge, and then $PGF_{2\alpha}$ concentration dropped to the basal level (12.5 pg/ml) toward 48 h [23]. This period is followed by the starting period (starting at 72 h after the LH surge) of the present study with developing CL, when the $PGF_{2\alpha}$ concentration in OVP was 43.0 pg/ml. Thus, the data suggest that active synthesis of $PGF_{2\alpha}$ in the
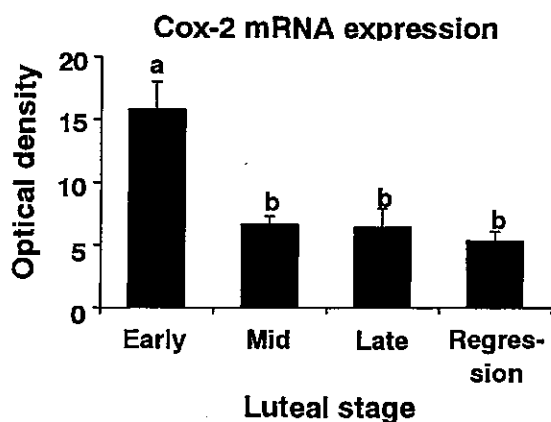
## Cox-2 mRNA expression



Fig. 4. Relative levels of Cox-2 mRNA (RT-PCR, 38 cycles, arbitrary units) in bovine CL during the estrous cycle. Results represent means ± SEM from 4 CL/ stage. Different superscript letters indicate significant differences (P<0.05).

present study was not a trace of the peak of PGF$_{2\alpha}$ that occurs during the periovulatory period, but a new peak during CL development.

The CL is filled with an enormous number of capillary vessels, and CL development is closely associated with angiogenesis. Angiogenic growth factors, bFGF and VEGF, have been shown to stimulate P synthesis within bovine early CL [4, 17] as well as neovascularization [1, 2]. Also, an acute increase of the luteal tissue area scanned by ultrasonography was observed from 3 days after ovulation [22]. Interestingly, the high PGs production observed for only short period in the present study synchronizes with the onset of the rapid development of CL. Furthermore, angiogenic growth factors stimulated PGF$_{2\alpha}$ and PGE$_2$ synthesis by bovine early CL *in vitro* [3, 4]. It has been also reported that bFGF increases prostaglandin H synthase activity [23] and VEGF induces constitutive Cox-1 expression in bovine endothelial cells [24]. Hence, the high PGs production during only the early stage of CL formation may be directly correlated with the stimulation of the growth of new capillary vessels by VEGF and bFGF, resulting in activation of PGs production within CL.

The rupture of the follicular wall at ovulation is characterized as an inflammatory reaction [25]. It is generally accepted PGs contribute importantly to the genesis of the symptoms of inflammation [26, 27]. Although PGs do not appear to have direct

effects on vascular permeability, PGE$_2$ markedly enhances leukocyte infiltration by promoting blood flow in the inflamed region [28]. In addition, Cox-2 is induced in settings of inflammation by cytokines and inflammatory mediators [29]. The immune cells within the CL have the ability to produce cytokines [30]. Tumor necrosis factor α (TNF-α) and interleukin-1β (IL-1β) have been shown to stimulate PGE$_2$ and PGF$_{2\alpha}$ synthesis by bovine luteal cells [31, 32], and the stimulatory effect of TNF-α on PGE$_2$ synthesis by early luteal cells is higher than at other stages [32]. IL-1β also increases the Cox-2 mRNA expression in cultured human luteinized granulosa cells [33]. Thus, the active production of PGE$_2$ as well as PGF$_{2\alpha}$ may be stimulated by both cytokines and angiogenic factors.

Active PGs production in the developing CL was confirmed by the present *in vivo* study as described above, and was supported by the Cox-2 mRNA expression result. The CL of this specific period has refractoriness to exogenous PGF$_{2\alpha}$ [34, 35]. In mid luteal phase, an injection of PGF$_{2\alpha}$ caused the decrease of mRNA encoding 3β-hydroxysteroid dehydrogenase (3β-HSD) and cytochrome P450 side chain cleavage (P450scc) within bovine CL [36, 37]. Likewise, a single injection of PGF$_{2\alpha}$ before Day 5 to a cow resulted in the reduction of the expression of 3β-HSD and PGF$_{2\alpha}$ receptor mRNA in a similar manner to that in the mid luteal phase [36, 37], but the P450scc mRNA expression was not affected [37]. Bovine CL after PGF$_{2\alpha}$ treatment before Day 5 of the estrous cycle grows normally, and does not regress. These findings suggest that the reason why the bovine early CL is resistant to exogenous PGF$_{2\alpha}$ is not due to the lack of binding sites for PGF$_{2\alpha}$ on endothelial and luteal cells, but different intracellular mechanism of PGF$_{2\alpha}$ action between early and aged CL. Importantly, the stimulatory effect of PGF$_{2\alpha}$ on P secretion has been reported both *in vivo* [38] and *in vitro* studies [4, 10–14]. PGF$_{2\alpha}$ has been shown to stimulate adenosine 3′, 5′-monophosphate (cAMP) in bovine luteal cells [13], and is capable of stimulating P production of luteinized granulosa and theca cells and elevating the mRNA expression for P450scc [39]. Similarly, PGE$_2$ also stimulates P synthesis by bovine CL *in vitro* [3, 14]. These lines of evidence suggest that the luteotropic action of PGs is partly dependent on cAMP activation.

Taken together, the present results provide direct

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