

Intraluteal regulation of prostaglandin F2 α -induced prostaglandin biosynthesis in pseudopregnant rabbits

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Abstract

The objective of the present study was to investigate in rabbit corpora lutea (CL), at both the cellular and molecular level, intraluteal cyclooxygenase (COX)-1, COX-2 and prostaglandin (PG) E2-9-ketoreductase (PGE2-9-K) enzymatic activities as well as *in vitro* PGE2 and PGF2 α synthesis following PGF2 α treatment at either early- (day-4) or mid-luteal (day-9) stage of pseudopregnancy. By immunohistochemistry, positive staining for COX-2 was localized in luteal and endothelial cells of stromal arteries at both the stages. In CL of both stages, basal COX-2 mRNA levels were poorly expressed, but rose ($P < 0.01$) 4- to 10-fold 1.5–6 h after treatment and then gradually decreased within 24 h. Compared to mid-stage, day-4 CL had lower ($P < 0.01$) COX-2 and PGE2-9-K basal activities, and PGF2 α synthesis rate, but higher ($P < 0.01$) PGE2 production. Independent of luteal stage, PGF2 α treatment did not affect COX-1 activity. In day-4 CL, PGF2 α induced an increase ($P < 0.01$) in both COX-2 activity and PGF2 α synthesis, whereas that of PGE2 remained unchanged. In day-9 CL, PGF2 α up-regulated ($P < 0.01$) both COX-2 and PGE2-9-K activities, and PGF2 α production, but decreased ($P < 0.01$) PGE2 synthesis. All changes in gene expression and enzymatic activities occurred within 1.5 h after PGF2 α challenge and were more marked in day-9 CL. Our data suggest that PGF2 α directs intraluteal PG biosynthesis in mature CL, by affecting the CL biosynthetic machinery to increase the PGF2 α synthesis in an auto-amplifying manner, with the activation of COX-2 and PGE2-9-K; this may partly explain their differentially, age-dependent, luteolytic capacity to exogenous PGF2 α in rabbits.

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Introduction

The corpora lutea (CL) are transient ovarian organs that play a critical role for the establishment and maintenance of pregnancy by secreting progesterone (Niswender *et al.* 2000). However, if pregnancy fails to occur, the CL undergo luteolysis, a dynamic regression process that ends with their complete functional and structural demise (McCracken *et al.* 1999).

It is now widely accepted that prostaglandins (PGs) play a key role in regulating the function and life span of CL. In fact, PGF2 α has been identified as the main luteolytic factor of uterine origin in several non-primate mammals including the rabbit (O'Grady *et al.* 1972, Keyes & Bullock 1974, Lytton & Poyser 1982), and PGE2 as an important luteoprotective factor with luteotrophic or antiluteolytic actions (Niswender *et al.* 2000). In many species, the CL themselves synthesize PGF2 α and PGE2 (Gobetti *et al.* 1999, Boiti *et al.* 2000, Diaz *et al.* 2002, Zerani *et al.* 2005), whose production is regulated by a

large array of local and systemic factors, suggesting a paracrine and autocrine role for these two PGs (Olofsson & Leung 1996, Davis & Rueda 2002, Diaz *et al.* 2002, Wiltbank & Ottobre 2003, Arosh *et al.* 2004, Boiti *et al.* 2005).

The critical step in PG biosynthesis is the enzymatic conversion of phospholipase A2-derived arachidonic acid into PGH2 by cyclooxygenase-1 (COX-1) or COX-2 (Smith *et al.* 1996, Sakurai *et al.* 2003, 2005, Simmons *et al.* 2004). PGH2, in turn, is converted into four structurally active PGs (PGE2, PGF2 α , PGD2 and PGI2) via specific PG synthases (Helliwell *et al.* 2004). However, the biosynthesis of PGF2 α is peculiar because it derives from three different pathways catalysed by corresponding ketoreductases using PGH2, PGD2 or PGE2 as substrates (Watanabe 2002). In rabbits, the PGE2-9-ketoreductase (PGE2-9-K) was found in the ovary (Schlegel *et al.* 1987) and more recently also in CL (Wintergalen *et al.* 1995).

Although in the past few years the down-stream mechanisms activated by exogenous PGF₂α received much attention in rabbits (Boiti *et al.* 1998, 2000, 2003, Gobbetti *et al.* 1999), the possible autocrine and/or paracrine roles of PGF₂α in this species are still poorly understood. Similarly, little is known about what mechanisms do protect the growing CL from functional luteolysis, which occurs in the early luteal stage until day 6 of pseudopregnancy, when CL shift from refractoriness to partial responsiveness to exogenous PGF₂α (Boiti *et al.* 1998).

Therefore, the main objective of this study was to compare, in the rabbit model, the key enzymes involved in the PGs biosynthesis, and their intraluteal modulation after induction of CL regression by means of PGF₂α administered at either early- or mid-luteal stage of pseudopregnancy, at days 4 and 9 respectively. With this end in view, experiments were devised to characterize the dynamic of gene expression patterns for luteal COX-2, its precise cell type localization within the ovary, as well as the *in vitro* rates of enzymatic activity for COX-1, COX-2 and PGE₂-9-K and for the synthesis of PGE₂ and PGF₂α on CL explanted at different time points up to 24 h after PGF₂α treatment.

Materials and Methods

Reagents

Random hexamer primers, DNase I (DNAase I Amp. Grade), RNase H- reverse transcriptase (Superscript II), *Escherichia coli* RNase H and DNA ladders were obtained from Invitrogen as well as reagent for isolation and purification of total RNA (TRIzol), Taq DNA polymerase (Platinum), RNase-free tubes and RNase-free water and deoxy-NTPs. Primers for 18S rRNA and corresponding competitors (QuantumRNA 18S Internal Standards) were acquired from Ambion (Austin, TX, USA), whereas primers for mRNAs of COX-2 were obtained from Invitrogen. Nucleospin Extract II kit for DNA extraction from agarose gels was from Macherey-Nagel (Düren, Germany). Tritiated hormones and arachidonic acid were purchased from Amersham Biosciences, while progesterone, PGF₂α and PGE₂ antisera, and non-radioactive hormones came from Sigma. The kit for the protein assay was purchased from Bio-Rad Laboratories. The primary polyclonal antibody goat anti-COX-2, used for immunohistochemistry was supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA), whereas the biotinylated secondary antibody, chicken anti-goat IgG and normal chicken serum were purchased from Santa Cruz Biotechnology. The avidin-biotin complex (ABC; Vector Elite Kit) and the chromogen 3,3'-diaminobenzidine tetrachloride (DAB) were from Vector Laboratories. Silica gel 60 were purchased from Baxter (Baxter Scientific Products,

McGaw Park, IL, USA), whereas all the other pure grade chemicals and reagents were obtained locally.

The following hormonal preparations were administered via i.m. injection: gonadotrophin-releasing hormone (GnRH) analogue (Receptal, Hoechst-Roussel Vet, Milan, Italy), Pregnant mares serum gonadotrophin (PMSG; Folligon, Intervet, Milan, Italy) and alfaprostol (Gabbrostim, Centralvet, Milan, Italy) a PGF₂α analogue.

Animals, hormonal regimen and luteal tissue collection

The protocols involving the care and use of the animals for these experiments were approved by the Bioethic Committee of the University of Perugia.

Unmated New Zealand White rabbits of 5-months age, weighing 3.5–3.8 kg, were caged individually in quarters of the University of Perugia Central Animal Facility and maintained under controlled conditions of light (14 h light:10 h darkness) and temperature (18 °C). The animals were provided commercial rabbit chow and drinking tap water *ad libitum*. All rabbits were treated with 20 IU of PMSG followed 3 days later by an i.m. injection of 0.8 µg of GnRH to induce pseudopregnancy (Stradaioli *et al.* 1997). The day of GnRH injection is designated day 0. This hormonal protocol was effective in inducing pseudopregnancy.

On day 4 or 9 of pseudopregnancy, the rabbits ($n=18$ /group) were administered i.m. 200 µg alfaprostol. At each luteal stage, three rabbits were killed by cervical dislocation just before (time 0) and then 1.5, 3, 6, 12 and 24 h after PGF₂α administration. Reproductive tracts, promptly removed from each animal, were thoroughly washed with saline. Within a few minutes, the CL were excised from ovaries and, after careful dissection of non-luteal tissue by fine forceps under stereoscopic magnification, immediately frozen at –80 °C, after rinsing with RNase-free PBS, for later evaluation of gene expression, or processed for *in vitro* determination of enzymatic activities and PG synthesis. For the immunohistochemical detection of COX-2, two additional animals for each time point were killed just prior (time 0), 1.5 and 3 h after PGF₂α injection administered at either day 4 or 9 of pseudopregnancy. The ovaries, immediately excised after killing, were fixed by immersion in 4% (w/v) formaldehyde in PBS (pH 7.4) for 24 h at room temperature, and subsequently processed for embedding in paraffin following routine tissue preparation procedures.

Progesterone plasma levels were used as a marker of luteal functional activity. From each rabbit, two blood samples were collected by venous puncture of the marginal ear vein, one just before PGF₂α treatment and the other immediately prior to killing. The samples, collected in EDTA vacutainers, were centrifuged at 3000 g for 15 min and plasma was stored frozen until assayed for progesterone concentrations to assess the functional status of the ovarian CL. For the purpose of this

work, functional luteolysis was defined as a 50% decrease in plasma progesterone from pre-treatment values, while complete luteolysis as the failure of CL to secrete progesterone so that blood levels fall below 1.0 ng/ml, which are found in oestrous rabbits (Browning *et al.* 1980).

Progesterone assay

Progesterone concentrations were determined by RIA, using specific antibody according to the procedure reported elsewhere (Boiti *et al.* 2000). Progesterone was extracted from corresponding 0.1 ml plasma samples with ethyl ether and each sample was assayed in duplicate. The assay sensitivity was 0.08 ng/ml for progesterone, whereas intra- and interassay coefficients of variations were 5.3 and 10.2% respectively.

Immunohistochemistry of COX-2

The immunohistochemical detection of COX-2 was performed using a modification of a previously reported procedure (Boiti *et al.* 2005). Briefly, serial 7 μm -thick sections, mounted on poly-L-lysine coated glass slides, were dewaxed in xylene and hydrated through graded ethanol; then they were microwaved for 5 min at 750 W in 10 mM citric acid (pH 6.0) for antigen retrieval. All subsequent steps were carried out in a moist chamber at room temperature. The sections were first treated with a 0.5% solution of hydrogen peroxide in methanol for 10 min, in order to inactivate the endogenous pseudo-peroxidase activity, then rinsed with PBS solution and incubated for 30 min with normal chicken serum to minimize the non-specific binding of reagent in subsequent steps. To reduce the variation in staining, within each luteal stage, all tissue sections were incubated together. The primary antiserum, goat anti-COX-2 antibody (sc-1747) was diluted 1:50 in PBS and left on sections overnight. The next day, the sections were washed in PBS and incubated with a biotinylated secondary antibody (1:200 in PBS) for 30 min. After PBS washes, sections were exposed to avidin-biotin-peroxidase complex (1:2500 in PBS) for 30 min, followed by the chromogen DAB for 5 min, to visualize the site of reaction. After washing in tap water, the ovary sections were dehydrated and mounted in Canada balsam natural. Positive reactions were recognized as reddish brown precipitates. Sections, in which the primary antibody was omitted or substituted by pre-immune goat gamma globulin, were used for the negative control of non-specific staining.

RNA extraction and RT

For each rabbit, the total RNA was extracted from a pool of eight CL, which was homogenized by Omni- μH mixer (Analytical Control, Dasit, Cinisello Balsamo, Milan,

Italy) in 1 ml solution provided with Trizol as previously described (Boiti *et al.* 2003). Concentration of total RNA (OD_{260}) and purity ($\text{OD}_{260/280}$, $\text{OD}_{260/230}$) were determined spectrophotometrically (BioPhotometer, Eppendorf srl, Milan, Italy). The integrity of each sample was assessed by electrophoresis of an aliquot of 3 μg RNA in agarose formaldehyde gel using ethidium bromide staining. Genomic DNA contamination was prevented by treatment with deoxyribonuclease I according to instructions. Five microgram of total RNA (1 $\mu\text{g}/\mu\text{L}$) was reverse transcribed into cDNA in a 20 μl final reaction mixture of iSCRIPT cDNA Synthesis Kit (Bio-Rad). Genomic DNA contamination was checked by carrying samples through PCR procedure without reverse transcriptase. The RT products were stored at -20°C .

Multiplex RT-PCR amplification

An aliquot (1.0 μl) of cDNA was used as a template for the subsequent semi-quantitative PCR amplification reaction. This (25.0 μl) was performed with 0.2 μl Taq DNA Polymerase (5 U/ μl), 1.0 μl dNTPs (10 mM), 5.0 μl Taq buffer 10 \times , 1.0 μl (10 μM) of both forward and reverse primers. The primer sequences were: COX-2, product size 121 bp, forward 5'-CCTCACTGATGGGCTGTTTT-3', reverse 5'-GGTGAAAGCAATGCCTGAAT-3'; 18S (accession n. 10098): product size bp: 489, forward 5'-TCAAGAACGAAAGTCGGAGGTT-3', reverse 5'-GGA-CATCTAAGGGCATCA-3'. The semi-quantitative PCRs were carried out as reported previously (Boiti *et al.* 2005). Preliminary experiments were carried out to establish the optimal ratio between 18S primers and their competitors. Between 30 and 40 cycles, both target and 18S products were in a linear exponential phase of amplification (data not shown). To minimize errors within each experiment, the target gene was co-amplified with housekeeping 18S primers at the same PCR cycle. The amplification was performed on a thermal cycler (GeneAmp, PCR System, Perkin-Elmer Biosystem, Foster City, CA, USA). All PCR consisted of a first denaturing cycle at 94°C for 75 s, followed by an amplification profile of 35 reaction cycles with a first denaturing cycle at 94°C for 15 s, followed by annealing at 60°C for 30 s and extension at 72°C for 45 s and a final extension step at 72°C for 10 min. Within each experiment, the complete set of samples was processed in parallel in a single PCR, using aliquots of the same PCR master mix. Each set of determinations was performed in triplicate.

Analysis of amplification products

The amplified PCR-generated products (20 μl of 25 μl total reaction volume) were analysed by electrophoresis on 2% agarose gel using ethidium bromide staining. One product for each time point was electrophoresed on a

single gel together with a negative control that contained no RNA and standard DNA ladder. The images of gels were acquired using a Kodak DC290 digital camera. The background-corrected band intensities (absolute optical densities with the background levels from corresponding lanes subtracted for each PCR product) were quantified using Quantity One software (Bio-Rad Laboratories). To evaluate the temporal changes in relative levels of mRNAs, the band intensities for the target genes of interest obtained from each aliquot of PCR products were normalized against those of the housekeeping 18S mRNA co-amplified product in the same aliquot. Values were expressed as arbitrary units of relative abundance of the specific target genes.

The amplified products, collected from agarose gel after electrophoresis, were purified with Nucleospin Extract II kit and their identity confirmed by DNA sequencing with Sanger's method.

COX-1 and COX-2 enzyme activity determination

Luteal COX-1 and -2 activities were determined by measuring the disappearance of the radiolabelled substrate [^3H]arachidonic acid using a modified method previously reported (Xu *et al.* 1997). Three to four CL of each rabbit were pooled and homogenized in 1 ml cold fresh buffer (50 mM Tris-HCl and 1 mM EDTA, pH 8.0), centrifuged at 20 000 *g* for 60 min at 4 °C, and the supernatant was used for subsequent determination of enzyme activity in duplicate. To each incubation tube 50 μl supernatant and 50 μl buffer were added, containing 150 000 d.p.m. [^3H]arachidonic acid (specific activity 150–230 Ci/mmol), either alone or with a selective COX-2 inhibitor (NS-398 1 μM). The sample and substrate mixture with a non-selective COX inhibitor (acetylsalicylic acid, 1 mM) was used to determine the [^3H]arachidonic acid disappearance values due to other enzymatic activities (lipoxygenase and/or epoxygenase) and non-enzymatic reactions (Parthasarathy *et al.* 1989). The mixture was then incubated at 37 °C for 30 min. Termination was achieved by addition of isopropanol/*n*-heptane/1 N sulphuric acid (1:4:0.1, v/v/v). The phases were separated by the addition of 400 μl H_2O and 1 ml *n*-heptane. The upper organic layer, containing the unreacted [^3H]arachidonic acid was transferred to a second tube and the aqueous layer was extracted again with 1 ml *n*-heptane. The organic layers were combined with an additional 1 ml *n*-heptane and approximately 150 mg silica gel 60. The mixture was thoroughly mixed and centrifuged. The clear supernatant was transferred to a scintillation vial and the amount of [^3H]arachidonic acid quantified by liquid scintillation counting. For each sample, COX-1 activity was determined by calculating the rate of loss of [^3H]arachidonic acid incubated with selective COX-2 inhibitor. Conversely, the COX-2 activity of each corresponding sample was determined by calculating

the rate of loss of [^3H]arachidonic acid incubated without selective COX-2 inhibitor, and subtracting from this value that of COX-1. The values for COX-1 and COX-2 were corrected by subtracting the [^3H]arachidonic acid disappearance values due to other enzymatic activities and non-enzymatic reactions. Preliminary evidence led to our choosing the incubation conditions and the minimum effective dose of COX-2 inhibitor used in the present *in vitro* study (Fig. 1).

PGE2-9-K enzyme activity determination

Luteal PGE2-9-K activity was determined by measuring the conversion of [^3H]PGE2 into [^3H]PGF2 α using a previously reported modified method (Gobbetti & Zerani 1995a). Briefly, each pool of CL was homogenized in 1 ml cold fresh homogenating buffer (20 mM K_2HPO_4 , 1 mM EDTA and 10 mM β -mercaptoethanol, pH 7.4). Total homogenate was filtered and immediately used for the assay of enzymatic activity. One hundred microlitres of homogenate and 50 μl of homogenating buffer containing 150 000 d.p.m. [^3H]PGE2 (specific activity 140–170 Ci/mmol) and NADPH (3 mg/ml) were added to the incubation tube. The mixture was incubated at 37 °C for 10 min. Termination was achieved by addition of 100 μl 0.1 M HCl. PGs were extracted with diethyl ether and resuspended with 500 μl RIA buffer (74.5 mM Na_2HPO_4 , 12.5 mM EDTA-Na, 0.1% gelatine, pH 7.5). Two hundred microlitres RIA buffer containing PGF2 α -specific antiserum were added to duplicated samples and the mixture was incubated at 4 °C for 16 h.

NS398 dose-response curve on COX2 activity

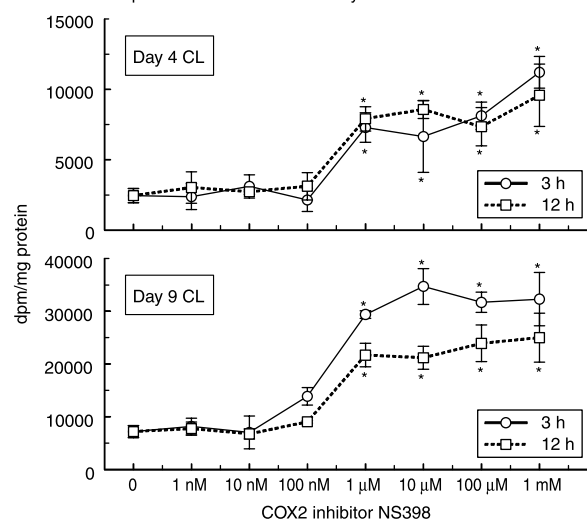


Figure 1 *In vitro* effects of increasing concentrations of COX-2 inhibitor (NS-398) on COX-2 activity by rabbit CL collected during early- and mid-luteal phases, at days 4 (upper panel) and 9 (lower panel) respectively, and incubated for 3 (circles) and 12 (squares) h. Each point represents mean \pm s.d. of three combined values. Asterisks indicate a significantly different value ($P < 0.01$).

The [^3H]PGF2 α -antiserum bound fraction was determined as previously indicated.

PGE2 and PGF2 α *in vitro* synthesis

Luteal PGE2 and PGF2 α *in vitro* synthesis were determined by measuring the conversion of the [^3H]arachidonic acid into [^3H]PGE2 and [^3H]PGF2 α , using a previously reported modified method (Gobbetti & Zerani 1995b). Fifty microlitres of supernatant, obtained by homogenizing each pool of CL as previously described, and 50 μl of incubation buffer (50 mM Tris, 1 mM EDTA and 1 mM EGTA, pH 7.4) containing 150 000 d.p.m. [^3H]arachidonic acid were added to the incubation tube. The sample and substrate mixture with a non-selective COX inhibitor (indomethacin 1 mM) was used as a blank. The mixture was incubated at 37 $^{\circ}\text{C}$ for 30 min and then stopped by addition of 100 μl 0.1 M HCl. PGs were extracted with diethyl ether and resuspended in 500 μl RIA buffer. Two hundred microlitres RIA buffer containing PGE2- or PGF2 α -specific antiserum were added to duplicated samples and the mixture was incubated at 4 $^{\circ}\text{C}$ for 16 h. The [^3H]PGE2- or [^3H]PGF2 α -antiserum-bound fractions were separated with charcoal-dextran suspension and the radioactivity was quantified by liquid scintillation counting.

Statistical analysis

At each stage of pseudopregnancy, the ratios of each PCR product for target COX-2 gene normalized against 18S co-amplified product were analysed by two way ANOVA, time after PGF2 α treatment and gel being the two sources of variability (Sokal & Rohlf 1981). Plasma progesterone levels, COX-1, COX-2 and PGE2-9-K enzyme activities as well as PGE2 and PGF2 α *in vitro* synthesis data were evaluated by ANOVA followed by Duncan's multiple range test (Duncan 1955). Correlation coefficients followed Sokal & Rohlf (1981).

Results

In vivo induction of luteolysis

Progesterone plasma concentration, used as a marker of luteal functional activity, decreased in rabbits 8 h after PGF2 α injection at day 9 of pseudopregnancy, and complete functional regression was achieved 24 h later when it declined to 0.3 ng/ml. As expected, PGF2 α was ineffective in inducing a functional regression when administered at day 4 of pseudopregnancy (Fig. 2).

COX-2 immunolocalization in rabbit ovary

Using a polyclonal antibody, immunostaining reaction for COX-2 was localized in different portions of un-treated (time 0) rabbit ovaries collected at days 4 (Fig. 3a–c) and 9

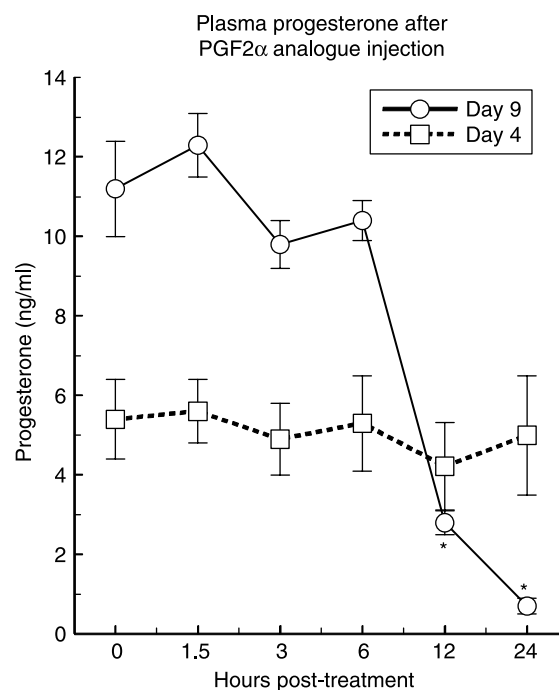


Figure 2 Plasma progesterone concentrations in blood samples collected at 0, 1.5, 3, 6, 12 and 24 h after PGF2 α analogue injection at days 4 or 9 of pseudopregnancy. Apart from time 0, each data point represents mean \pm s.e.m. of values derived from three different rabbits. Asterisks indicate a significantly different value ($P < 0.01$).

(Fig. 4a–c) of pseudopregnancy, including CL parenchyma (a, Figs 3 and 4), vascular components (b, Figs 3–5) and ovarian epithelium (c, Figs 3 and 4). In each positive cell type, staining was also evident 1.5 and 3 h after PGF2 α injection, independently of luteal stage at days 4 and 9 (data not shown). Staining was completely abolished when the primary antibody was substituted with non-immune serum (d–f, Figs 3 and 4).

Gene expression of mRNA for luteal COX-2

The corresponding base pair amplification products obtained using primer designed for the COX-2 gene matched the expected sizes (Fig. 6, panels, B and C). Sequence analysis showed that the 121 bp PCR products were homologue to the published sequence of COX-2 cDNA for rabbits.

In un-stimulated rabbits, the relative COX-2 mRNA level was higher ($P < 0.01$) in mid- than in early phase CL (Fig. 6, panel A). At day 4 of pseudopregnancy, COX-2 mRNA transcript sharply increased tenfold ($P < 0.01$) already 1.5 h following PGF2 α treatment and remained at approximately this level for the next 24 h (Fig. 6, panel A). At day 9 of pseudopregnancy, the relative abundance of COX-2 mRNA rose threefold 1.5 h after PGF2 α administration and was fourfold ($P < 0.01$) more expressed 3 and 6 h later; thereafter, COX-2 mRNA levels gradually decreased within the following 24 h, remained higher ($P < 0.01$) than pre-treatment basal

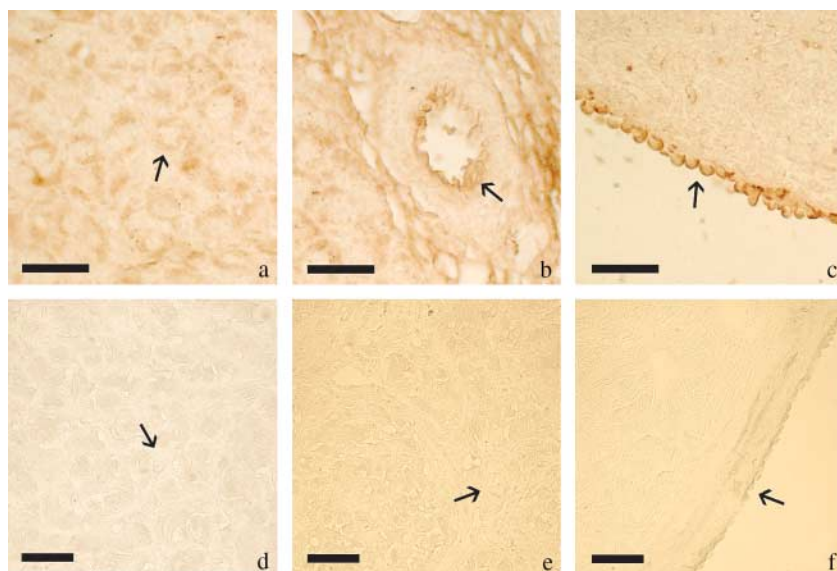


Figure 3 Cellular immunolocalization of COX-2 in rabbit ovaries collected at day 4 of pseudopregnancy before (a–c) PGF2 α analogue injection. In photos a–c, the arrows indicate the sites of positive immunoreaction: CL parenchima (a), vascular components of stromal arterioles (b), ovarian epithelium (c). Control sections in the absence of primary antibody (d–f). Scale bar = 20 μ m.

values (Fig. 6, panel A). After PGF2 α treatment, COX-2 mRNA were more expressed ($P < 0.01$) at 3 and 6 h in day-9 than in day-4 CL (Fig. 6, panel A).

COX-1 and COX-2 enzyme activity

In un-stimulated does, basal luteal enzyme activity for COX-1 was similar at both stages of pseudopregnancy (Fig. 7, left panel), whereas that of COX-2 was higher ($P < 0.01$) at day 9 than at day 4 (Fig. 7, right panel). In the early luteal stage, basal COX-1 enzyme activity (7464 ± 1573 d.p.m./mg protein) was higher ($P < 0.01$) than that of COX-2 (3129 ± 1006 d.p.m./mg protein), while there was no difference in CL collected at day 9. Following PGF2 α challenge, the activity of COX-1 did not change neither at day 4 nor at day 9, whereas that of COX-2 increased ($P < 0.01$) from 1.5 to 24 h in day-4 CL

and from 1.5 to 12 h in day-9 CL, when it decreased to basal level (Fig. 7, left panel). The enzymatic activity rate of COX-2 after PGF2 α was much higher ($P < 0.01$) in day-9 than in day-4 CL (Fig. 7, right panel).

PGE2-9-K enzyme activity

Basal luteal PGE2-9-K activity was lower ($P < 0.01$) at day 4 than at day 9 of pseudopregnancy (Fig. 8). PGF2 α injection increased ($P < 0.01$) PGE2-9-K activity from 1.5 to 12 h in day-9 CL, while it was ineffective in early CL (Fig. 8).

PGE2 and PGF2 α in vitro synthesis

At day 4 of pseudopregnancy, intraluteal basal synthesis of PGE2 was higher ($P < 0.01$), while that of

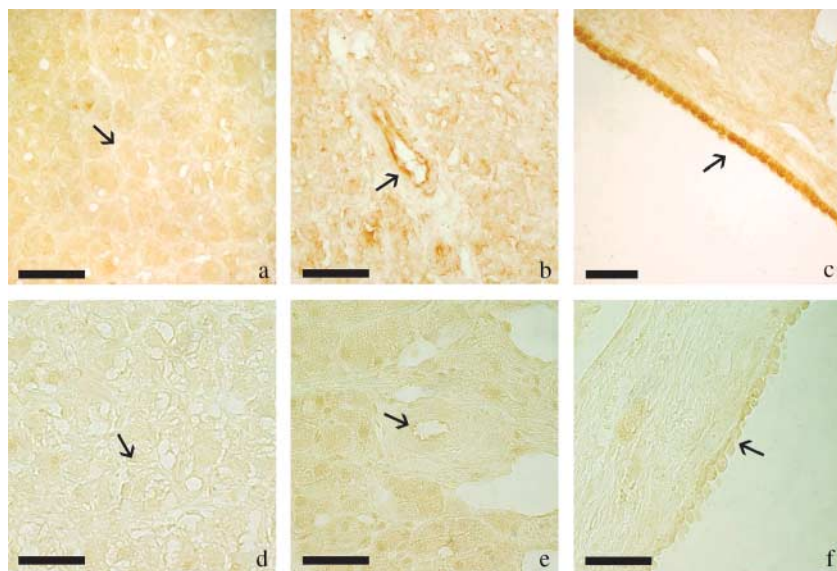


Figure 4 Cellular immunolocalization of COX-2 in rabbit ovaries collected at day 9 of pseudopregnancy before (a–c) PGF2 α analogue injection. In photos a–c, the arrows indicate the sites of positive immunoreaction: CL parenchima (a), vascular components of stromal arterioles (b), ovarian epithelium (c). Control sections in the absence of primary antibody (d–f). Scale bar = 20 μ m.

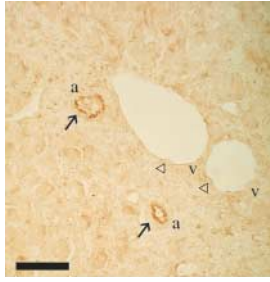


Figure 5 Positive COX-2 immunoreaction in rabbit stromal arterioles (a; arrows); in the same section the venules appeared negative (v; heads of arrows). The section was obtained from an ovary collected at day 4 of pseudopregnancy 3 h after PGF2 α injection. Scale bar=20 μ m.

PGF2 α was lower ($P<0.01$) compared with those at day 9 (Fig. 9). The PGE2 to PGF2 α basal synthesis ratio was higher ($P<0.01$) at day 4 (11.49 ± 3.45) than at day 9 (3.10 ± 0.66).

In the early luteal stage, exogenous PGF2 α administration did not affect the enzyme-dependent PGE2 luteal synthesis in the following 24 h, which markedly decreased ($P<0.01$) at day 9 of pseudopregnancy (Fig. 9, left panel). On the contrary, following PGF2 α challenge, intraluteal PGF2 α synthesis increased ($P<0.01$) within 1.5 h in both day-4 and day-9 CL, but was much higher ($P<0.01$) in day-9 than in day-4 CL (Fig. 9, right panel). PGE2 synthesis was negatively correlated with that of PGF2 α ($\nu=18$, $r=-0.8093$, $P<0.01$) and with PGE2-9-K activity ($\nu=18$, $r=-0.7135$, $P<0.01$); conversely, PGF2 α was positively correlated with PGE2-9-K activity ($\nu=18$, $r=0.8618$, $P<0.01$).

Discussion

In this study, we present evidence of an auto-amplification mechanism within the rabbit CL for PGF2 α synthesis that implies activation of COX-2 and PGE2-9-K enzymes and leads to luteal regression.

Our investigation revealed a widespread distribution of COX-2 immunostaining that was localized in several cell types within the rabbit ovary at both day 4 and 9 of pseudopregnancy either before or after exogenous PGF2 α injection. Similar distribution of COX-2 has been recently described in the human ovary (Stavreus-Evers *et al.* 2005). In bovine CL, across the oestrous cycle, independently of luteal stages, COX-2 protein is localized in large luteal cells, but not in other cell types (Arosh *et al.* 2004). In non-pregnant bitches, immunohistochemistry localized COX-2 expression in the cytoplasm of luteal cells during early dioestrus, but not during late ones (Kowalewski *et al.* 2006). On the contrary, in pseudopregnant rats COX-2 immunolabelling was seen in luteal steroid-producing and interstitial cells, but predominantly in non-luteal cells (Arend *et al.* 2004). In our study, positive COX-2 immunoreactivity was also evident in the ovarian surface epithelial cells, which are the site of an inflammatory-like response during ovulation (Rae *et al.* 2004).

Following PGF2 α challenge, luteal COX-2 mRNA expression was markedly up-regulated within 1.5 h at both day 4 and 9 of pseudopregnancy. However, thereafter the dynamic expression pattern of COX-2 transcript was different between early- and mid-luteal stages. In fact, in early CL the COX-2 mRNA levels remained at the same relatively high values, while in mature CL they peaked 3–6 h after PGF2 α injection to

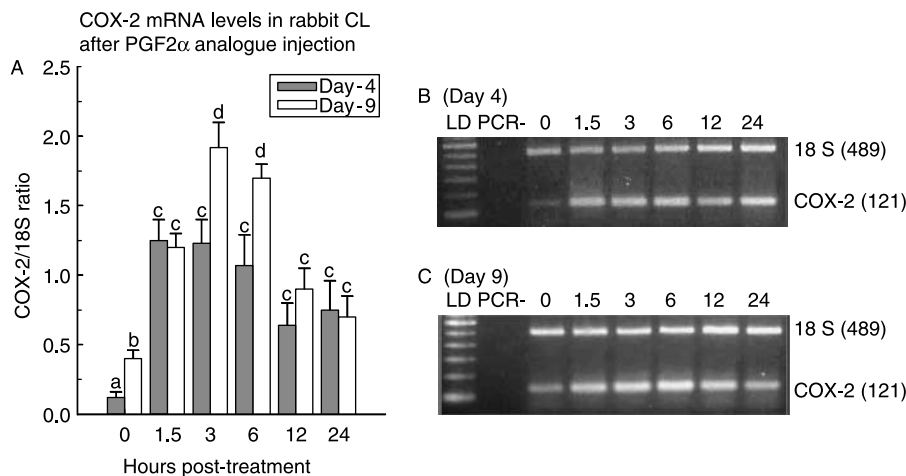


Figure 6 Gene expression patterns of COX-2 mRNAs in CL of rabbits collected at 0, 1.5, 3, 6, 12 and 24 h following PGF2 α analogue injection at days 4 or 9 of pseudopregnancy. Panel A summarizes the data (means \pm S.E.M.) derived from densitometric analyses of COX-2 in CL reported in arbitrary units relative to 18S expression. For each day of pseudopregnancy, and for each time point, the values combine the results from three different rabbits. Different letters above bars indicate a significantly different value ($P<0.01$). Panels B (day 4) and C (day 9) show representative photographs of typical 2% agarose, ethidium bromide stained gels, showing the presence of the expected base pair products yielded after RT-PCR using primers for target COX-2 (121 bp) and 18S (489 bp). Lane LD is the kilobase DNA marker, lane PCR represents a negative control of non-reverse-transcribed RNA submitted to PCR amplification, while the other lanes identify the corresponding hours after PGF2 α injection.

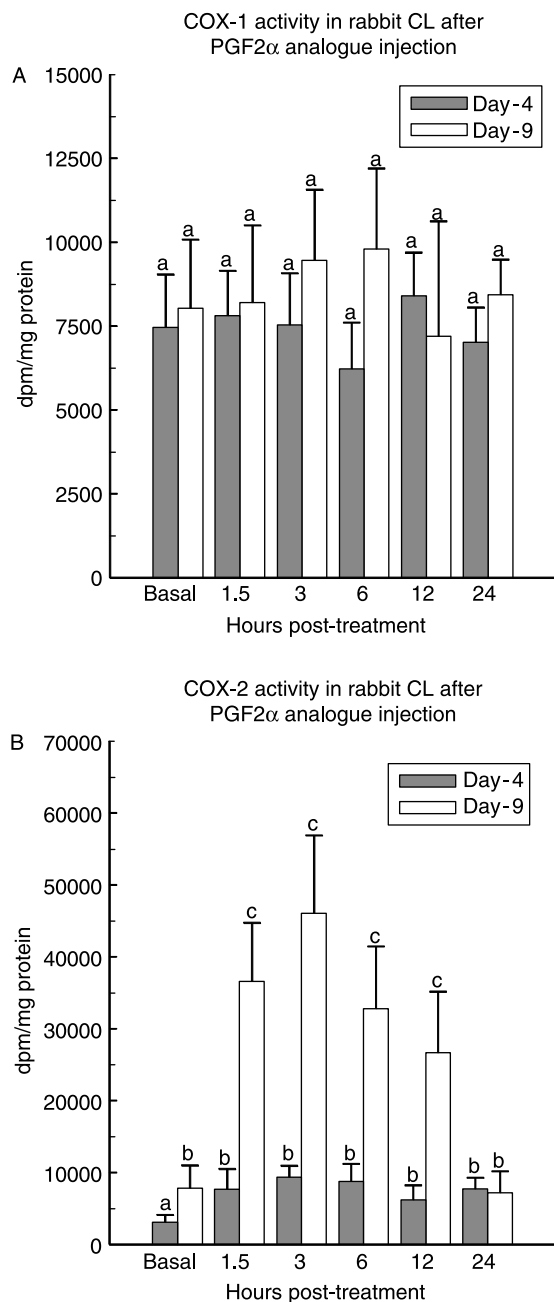


Figure 7 COX-1 (panel A) and COX-2 (panel B) activities in lysates of CL collected before and 1.5, 3, 6, 12 and 24 h after PGF2 α analogue administration at days 4 and 9 of pseudopregnancy. For each day of pseudopregnancy and for each time point, results are means \pm s.d. of combined values derived from three rabbits. Different letters above the bars indicate significantly different values ($P < 0.01$).

gradually decline within the following hours to low levels, but still higher with respect to pre-treatment ones. The results of the present study are in agreement with those of Narayansingh *et al.* (2002), who reported that in immature superovulated rats the administration of PGF2 α increased the luteal expression for COX-2

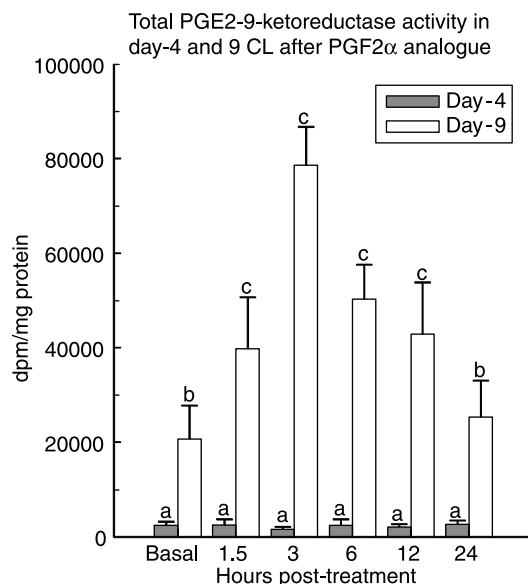


Figure 8 PGE2-9-K activities in lysates of CL collected before and 1.5, 3, 6, 12 and 24 h after PGF2 α analogue administration at days 4 and 9 of pseudopregnancy. For each day of pseudopregnancy and for each time point, results are means \pm s.d. of combined values derived from three rabbits. Different letters above the bars indicate significantly different values ($P < 0.01$).

mRNA and protein, whereas COX-1 remained unchanged. Also in cows, COX-2 mRNA expression increased during the time course of PGF2 α -induced luteolysis (Hayashi *et al.* 2003), while the COX-1 mRNA and related protein were constant during the different phases of the oestrous cycle (Arosh *et al.* 2004). In the dog, unlike other domestic animal species, luteal function is almost identical in pregnant and non-pregnant females and, in addition, PGF2 α seems to act as an endogenous luteolytic agent only during the immediate pre-partum decline in pregnant bitches (Hoffmann *et al.* 1999). In non-pregnant bitches, increased COX-2 expression was associated with luteal growth and not with CL regression, while COX-1 was constant during the dioestrus, suggesting that the COX-1 in generating prostanoids has a minor role in canine CL function (Kowalewski *et al.* 2006).

Our study revealed that the two luteal COX enzymes had different activity rates depending on the luteal stage examined. In fact, in both early- and mid-luteal phases of pseudopregnancy, COX-1 had the same activity, which was not affected by PGF2 α injection within the following 24 h. On the contrary, basal COX-2 activity was higher in day-9 than in day-4 non-stimulated CL. In addition, PGF2 α increased its specific activity two- to threefold during the early luteal phase and by five- to tenfold in mid-phase.

Regarding the intraluteal PG production, early stage CL, explanted from non-stimulated pseudopregnant rabbits, synthesized more PGE2 than older, mid-luteal

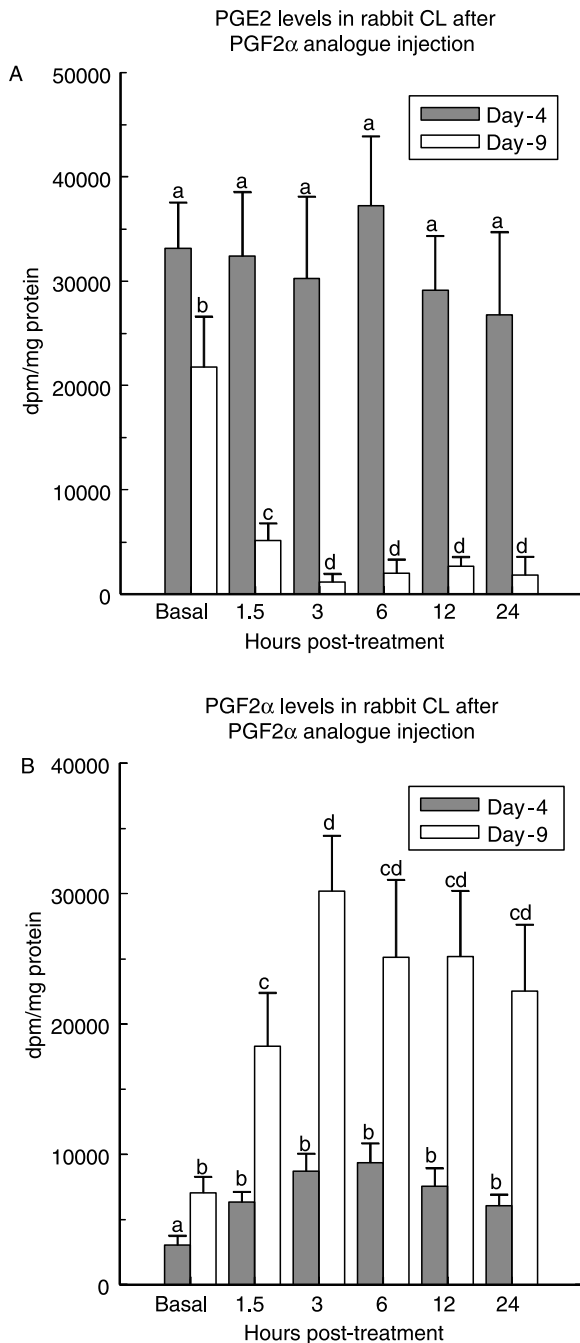


Figure 9 PGE2 (panel A) and PGF2 α (panel B) *in vitro* levels in lysates of CL collected before and 1.5, 3, 6, 12 and 24 h after PGF2 α analogue administration at days 4 and 9 of pseudopregnancy (left and right panels respectively). PGE2 and PGF2 α were determined by measuring the conversion of the [3 H]arachidonic acid into [3 H]PGE2 and [3 H]PGF2 α , respectively. For each day of pseudopregnancy and for each time point, results are means \pm s.d. of combined values derived from three rabbits. Different letters above the bars indicate significantly different values ($P < 0.01$).

stage CL, while day-9 CL produced PGF2 α at a higher level compared with day-4 CL. Despite the different approach, our results are in good agreement with those reported by Arosh *et al.* (2004), who found that in

growing CL of cows the expression of PGE2 synthase (PGES) protein was higher, whereas that of PGF synthase (PGFS) was lower. These authors reported that also the PGES/PGFS ratio was higher in growing CL of cows, in agreement with what we found in rabbits where the PGE2/PGF2 α ratio is higher in early luteal stage CL. In our animal model, after PGF2 α challenge, luteal PGE2 synthesis remained unaffected at day 4, but markedly decreased at day 9. On the contrary, following PGF2 α injection, intraluteal PGF2 α synthesis was much higher at day 9 than at day 4. These results are in agreement with our previous findings, where CL of rabbits were resistant to the luteolytic action of PGF2 α analogue, alfaprostol, injected at day 4 of pseudopregnancy, but acquired luteolytic capacity at day 9 (Boiti *et al.* 1998). A variety of species develops luteolytic capacity at different stages of the oestrous cycle, such as cows (Niswender *et al.* 2000), pigs (Guthrie & Polge 1976), mares (Douglas & Ginther 1975) and monkeys (Michael & Webley 1993). In cows, the lack of luteolytic capacity in developing CL (days 1–5) is not due to low PGF2 α receptor number, because these receptors are already highly expressed at this stage (Tsai & Wiltbank 1998), but rather to greater luteal PG dehydrogenase activity and lower PGF2 α synthetic capacity (Silva *et al.* 2000). In pigs, a single PGF2 α injection did not cause luteolysis prior to day 13 of the cycle (Diaz & Wiltbank 2005). In this species, the lack of luteolytic capacity is not due to an absence of PGF2 α receptors on luteal cells or to a lack of physiological response to this PG, but to different responsiveness of DAX-1 (a member of the orphan nuclear receptor family), StAR protein and LH receptor to PGF2 α (Diaz & Wiltbank 2005). In rabbits, the difference in luteolytic capacity during the luteal phases was explained with the different CL densities of PGF2 α receptors that increase four- to fivefold from early- to mid- and late-luteal phases (Boiti *et al.* 2001). We cannot exclude that the high PGE2/PGF2 α ratio, found in day-4 CL, is another explanation for the lack of luteolytic capacity in response to PGF2 α .

The PGF2 α synthesis can occur through the conversion of PGH₂, PGD₂ or PGE₂, by the enzymatic activity of PGH₂-9,11-endoperoxide ketoreductase, PGD₂-11-ketoreductase, or the PGE₂-9-K respectively (Watanabe 2002). However, the latter enzyme has a key role because its activity rate modulates PGE2 and PGF2 α production levels. In our study, mid-luteal phase CL showed a higher level of PGE₂-9-K basal activity than growing CL. PGF2 α injection increased the activity of this enzyme only in day-9 CL. The modulator role of PGE₂-9-K enzyme in rabbit CL is supported by the correlation of its enzymatic activity with PGE2 and PGF2 α synthesis, negative and positive respectively. Wintergalen *et al.* (1995) found that in rabbits, luteal PGE₂-9-K exerts also 20 α -hydroxysteroid dehydrogenase (20 α -HSD) catalytic activity, thus favouring the conversion of progesterone into the inactive metabolite

20 α -OH-progesterone. In regards to this double enzymatic activity, Madore *et al.* (2003) reported that a 20 α -HSD is present in the bovine endometrium and that this enzyme also has an aldose reductase activity and, thus, has the ability to terminate the oestrous cycle metabolizing progesterone and synthesizing PGF2 α . Therefore, an essential role emerges for PGE2-9-K in the regulation of induced-luteolysis in rabbits, as it increases intraluteal PGF2 α while decreasing progesterone production in PGF2 α -responsive CL.

The lack of effect of PGF2 α on luteal PGE2-9-K activity in day-4 CL could partly explain the modest PGF2 α -induced intraluteal PGF2 α production increase at this luteal stage, a result that is in line with the lack of luteolytic capacity in early CL to this PG. Since PGE2-9-K is not affected during the PGF2 α -induced luteolysis in the early phase of pseudopregnancy, the intraluteal increase in PGF2 α production is likely due to PGH2-9,11-endoperoxide ketoreductase and/or PGD2-11-ketoreductase. On the other hand, the absence of response of PGE2-9-K activity could explain, not only

the low receptor number for PGF2 α (Boiti *et al.* 2001) but also PGF2 α ineffectiveness in inducing progesterone decrease during the early luteal phase.

Recently, it was suggested that extra-luteal production of PGF2 α initiates the inhibition of progesterone production accompanying functional luteolysis, while the intraluteal PGF2 α production contributes to structural luteolysis by the activation of an auto-amplification loop in CL of pig, sheep and cow (Diaz *et al.* 2000, Griffith *et al.* 2002, Wiltbank & Ottobre 2003, Arosh *et al.* 2004). A positive feedback loop triggered by PGF2 α -FP receptor signalling was also found in endometrial adenocarcinoma cells; intracellular PGF2 α , produced via the COX-2 action, is actively transported outside the cell and interacts with FP receptor in an autocrine/paracrine manner activating the intracellular signalling cascade to sustain endometrial tumorigenesis (Jabbour *et al.* 2005).

Our results show that also in rabbits, intraluteal PGF2 α intervenes in the luteolysis with an auto-amplification loop as summarized in Fig. 10. Depending on luteal

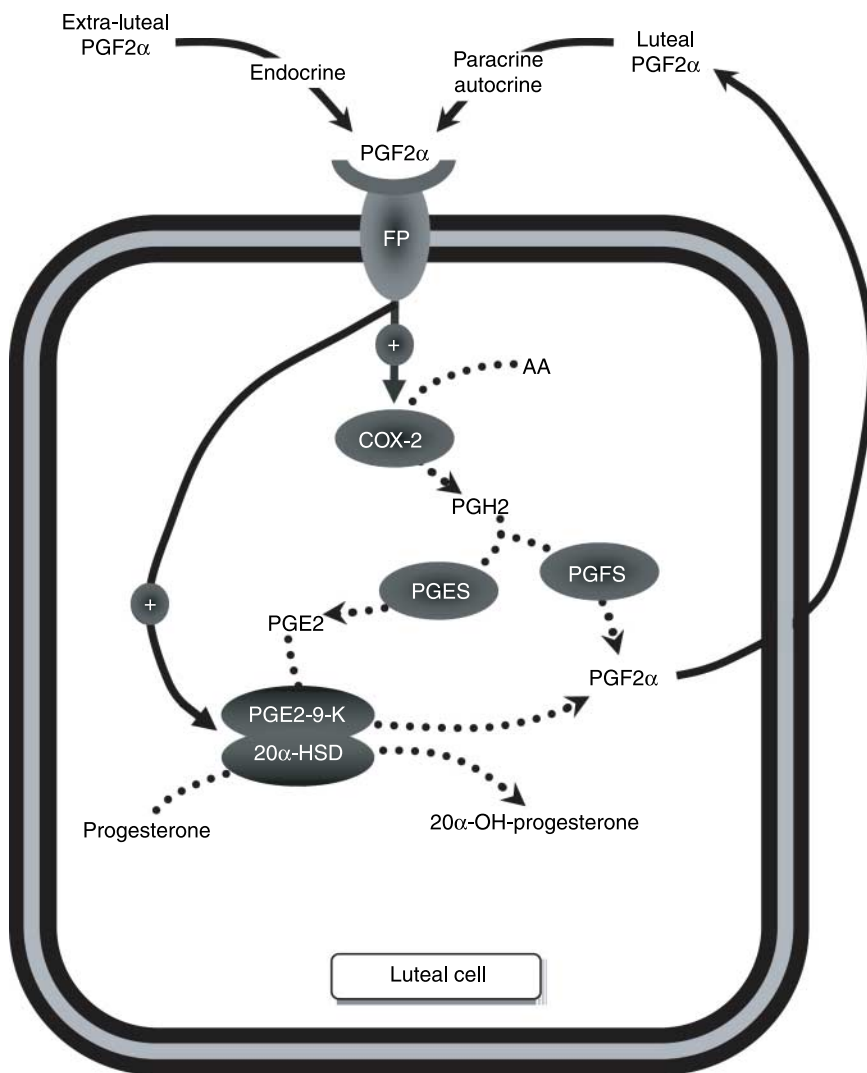


Figure 10 Schematic representation of the PF2 α auto-amplifying intracellular mechanisms in mid-luteal phase CL of pseudopregnant does. 20 α -HSD, 20 α -hydroxysteroid dehydrogenase; AA, arachidonic acid; COX-2, cyclooxygenase-2; FP, PGF2 α receptor; PGE2, prostaglandin E2; PGE2-9-K, PGE2-9-ketoreductase; PGES, prostaglandin E2 synthase; PGF2 α , prostaglandin F2 α ; PGFS, prostaglandin F2 α synthase; PGH2, prostaglandin H2. PGE2-9-K and 20 α -HSD are joined as they represent a single enzyme with two different activities. PGFS represents the activity of two enzymes: PGH2-9,11-endoperoxide ketoreductase and PGD2-11-ketoreductase.

stage, PGF2 α activates COX-2 and PGE2-9-K, the former enzyme metabolizes the arachidonic acid into PGH2, which is converted into PGF2 α and PGE2; this latter PG, in turn, is transformed into PGF2 α by the activated PGE2-9-K. This enzyme contemporaneously helps the PGF2 α -induced progesterone decrease, metabolizing progesterone into 20 α -OH-progesterone by its 20 α -HSD activity.

Recently, Wiltbank & Ottobre (2003) reported that luteal PGE2 production rose during luteolysis in ewes, and that this production was highly correlated to that of PGF2 α . These authors asserted that both PG productions were mediated by the increased COX-2 activity during luteolysis, but also that this intraluteal PGE2 increase did not have a clear physiological function during luteolysis, since this PG has a luteal protective effect. Our results suggest a physiological role for late-luteal phase PGE2 production; in fact, this PG could be the main source of PGF2 α synthesis throughout the PGE2-9-K enzymatic activity.

In a recent study, it was proposed that luteal tissue PG biosynthesis is preferentially directed toward PGE2 rather than PGF2 α production (Arosh *et al.* 2004), since the conversion of PGH2 to PGE2 by PGE synthase is 150-fold higher than the conversion of PGH2 to PGF2 α by PGF synthase (Madore *et al.* 2003, Thoren *et al.* 2003). These studies together with our present results suggest an intriguing idea: both early- and mid-luteal stage rabbit CL utilize the same intracellular enzymatic pathways (PLA2/AA \rightarrow COX2/PGH2 \rightarrow PGE synthase/PGE2) to produce an initial PGE2 bulk, while the final CL production of PGE2 (early luteal stage) or PGF2 α (mid-luteal stage) depends on PGE2-9-K inactivation or activation respectively.

Even if the present data throw new light on the knowledge of some physiological mechanisms regulating intraluteal PG production, further studies are needed to better understand the fine tuning that controls rabbit CL life span.

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