BIOLOGY OF REPRODUCTION (2013) **88**(3):62, 1–9
Published online before print 30 January 2013.
DOI 10.1095/biolreprod.112.107383

Evidence for a Luteotropic Role of Peroxisome Proliferator-Activated Receptor Gamma: Expression and In Vitro Effects on Enzymatic and Hormonal Activities in Corpora Lutea of Pseudopregnant Rabbits[1]

Massimo Zerani,[2,3] Margherita Maranesi,[4] Gabriele Brecchia,[4] Anna Gobbetti,[5] Cristiano Boiti,[4] and Francesco Parillo[3]

[3]*Scuola di Scienze mediche veterinarie, Università di Camerino, Matelica, Italy*
[4]*Laboratorio di Biotecnologie fisiologiche, Sezione di Fisiologia veterinaria, Dipartimento di Scienze biopatologiche ed Igiene delle produzioni animali e alimentari, Università di Perugia, Perugia, Italy*
[5]*Scuola di Bioscienze e biotecnologie, Università di Camerino, Camerino, Italy*

ABSTRACT

The expression of peroxisome proliferator-activated receptor gamma (PPARgamma) and its role in corpora lutea (CL) function were studied in pseudopregnant rabbits. Corpora lutea were collected at an early stage (Day 4), midstage (Day 9), and late stage (Day 13) of pseudopregnancy. Immunohistochemistry found evidence for the presence of PPARgamma in the perinuclear cytoplasm and nucleus of all the luteal cells; immunoreactivity decreased from the early to the late stage, with immunonegativity of the nuclei of late stage CL. *PPARgamma* mRNA transcript was expressed in all the luteal stages with the lowest level in the late stage. In CL cultured in vitro, the PPARgamma agonist (15-deoxy delta12,14 prostaglandin J2 [15d-PGJ2], 200 nM) increased and the antagonist (T0070907, 50 nM) decreased progesterone secretion at early and midluteal stages, whereas 15d-PGJ2 reduced and T0070907 increased PGF2alpha at the same stages. Prostaglandin-endoperoxide synthase 2 (PTGS2) activity was reduced by 15d-PGJ2 and increased by T0070907 in CL of early and midluteal stages. Conversely, 15d-PGJ2 increased and T0070907 reduced 3beta-hydroxysteroid dehydrogenase (3beta-HSD) activity in early and midluteal stage CL. PGE2 in vitro secretion as well as PTGS1 and 20alpha-HSD enzymatic activities were not affected by 15d-PGJ2 and T0070907 in any CL types. These results indicate that PPARgamma plays a luteotropic role in pseudopregnant rabbits, through PTGS2 down-regulation and 3beta-HSD up-regulation, with a consequent PGF2alpha decrease and progesterone increase.

3beta-HSD, corpora lutea, PGF2alpha, PPARgamma, progesterone, PTGS2, rabbit

INTRODUCTION

The peroxisome proliferator-activated receptors (PPARs), a family of three (α, δ, and γ) nuclear receptor/transcription factors, are involved in several processes such as steroidogenesis, angiogenesis, tissue remodeling, cell cycle, and apoptosis [1]. These processes are critical for normal ovarian function [2], and all three PPARs have been identified in the ovary of many species [3]. In particular, these nuclear receptors have been localized in the ovary of the rat [2, 4], mouse [5], pig [6], sheep [7], cow [8–10], rabbit [11], and human [12, 13]. Several in vitro studies have shown that PPAR agonists present contradictory actions on granulosa and luteal steroid secretion, depending on the species [1]. PPAR mechanisms in ovarian functions are not fully understood. However, comparisons with other cell models suggest that PPARs may regulate the genes required for follicle and corpora lutea (CL) maintenance [1].

It is widely accepted that prostaglandins (PGs) play a key role in regulating CL function and life span [14]. While PGF2α is the main luteolytic factor, PGE2 is an important luteoprotective factor with luteotrophic or antiluteolytic actions [15–17]. The critical step in PG biosynthesis is the enzymatic conversion of arachidonic acid (AA) into PGH2 by prostaglandin-endoperoxide synthase 1 (PTGS1) or PTGS2. PGH2, in turn, is converted into four structurally active PGs (PGE2, PGF2α, PGD2, and PGI2) via specific PG synthases [18]. However, the biosynthesis of PGF2α is peculiar because it derives from three different pathways catalyzed by corresponding ketoreductases using PGH2, PGD2, or PGE2 as substrates. In many species, the CL themselves synthesize PGF2α and PGE2, whose production is regulated by a large array of local and systemic factors, suggesting a paracrine and autocrine role for these two PGs [19–22].

In cardiac myocytes and in prostate cells, PPARγ was found to down-regulate the genes encoding PTGS2 [23], an enzyme implicated in the control of ovulation and CL life span [16]. Current research is devoting considerable attention to PGD2 and PPAR; in fact, an initial screening of AA metabolites revealed that a dehydration product of PGD2, namely the cyclopentanone PG, 15-deoxy delta12,14 prostaglandin J2 (15d-PGJ2), is a potent PPARγ ligand [2, 24]. A-series cyclopentanone PGs (PGAs) are dehydration products of PGE2 and can bind and activate PPARδ or PPARγ. PGAs are not considered a major product in the body but, conceivably, may be present in sites where PGE2 is produced [25]. PGF2α shows no binding to or activation of PPARs; however, PGF2α does indirectly inhibit PPARγ activity by mediating its phosphorylation [25].

The rabbit CL is a good model for investigating PPARγ mechanisms that regulate luteal function because in this species it is possible to induce pseudopregnancy by exogenous gonadotropin-releasing hormone (GNRH) administration [26].

[1]Supported by Regional Government of Umbria (Programma Operativo Regionale, FSE "Obiettivo Competitività Regionale e Occupazione" 2007–2013) and by the generous donations of the BC Red-water (Perugia, Italy) and PFZM Kitchenbrown (Matelica, Italy) Trusts.
[2]Correspondence: M. Zerani, Scuola di Scienze mediche veterinarie, via Circonvallazione 93, 62024 Matelica MC, Italy.
E-mail: massimo.zerani@unicam.it

Received: 21 December 2012.
First decision: 17 January 2013.
Accepted: 24 January 2013.

eISSN: 1529-7268 http://www.biolreprod.org
ISSN: 0006-3363

Therefore, the main objective of the present study was to examine the modulatory effects of PPARγ on rabbit CL during early, mid-, and late luteal stages of pseudopregnancy. With this end in view, experiments were devised to clarify the immunopresence and the gene expression of PPARγ, as well as its effects in vitro on the production of progesterone, PGE2, and PGF2α, and on the activity of the enzymes involved in the regulation of CL life span: 3β-hydroxysteroid dehydrogenase (3β-HSD), 20α-HSD, PTGS1, PTGS2, and PGE2-9-ketoreductase (PGE2-9-K).

MATERIALS AND METHODS

Reagents and Hormones

Mouse monoclonal anti-PPARγ (sc-7273) and anti-3β-HSD primary antibodies (sc-100466) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Biotin goat anti-mouse immunoglobulin G (IgG) secondary antibody, avidin-biotin complex (ABC, Vector Elite Kit), mouse IgG, and the chromogen 3,3′-diaminobenzidine tetrachloride (DAB) were obtained from Vector Laboratories (Burlingame, CA). The endogenous PPARγ agonist 15d-PGJ2 and the selective antagonist T0070907 (>800-fold selectivity for PPARγ over-PPARα and PPARδ) [27] were purchased from Tocris Bioscience (Bristol, U.K.).

Random hexamer primers, deoxyribonuclease I (DNAase I amplification grade), RNAse H-reverse transcriptase (Superscript III Reverse Transciptase), *Escherichia coli* RNase H, DNA ladders, the reagent for the isolation and purification of total RNA (TRIzol), Taq DNA polymerase (Platinum), RNAse-free tubes and water, and deoxynucleotide monophosphates were obtained from Invitrogen (Milan, Italy). Primers for *18S* rRNA and the corresponding competimers (QuantumRNA 18S internal standards) and *PPARγ* were also acquired from Invitrogen. Tritiated hormones and AA were purchased from Amersham Biosciences (Little Chalfont, Bucks, U.K.), while progesterone, PGF2α, and PGE2 antisera, and nonradioactive hormones (NS-398, trilostane, and R-flurbiprofen) came from Sigma (St. Louis, MO).

Incubation wells were obtained from Becton Dickinson Co. (Clifton, NJ), while medium 199 and Earle Balanced Salt Solution were from GIBCO (Grand Island, NY). HEPES and bovine serum albumin (BSA) were purchased from Sigma, whereas all other pure grade chemicals and reagents were obtained locally.

The following hormonal preparations were administered via intramuscular injection: equine chorionic gonadotropin (Folligon; Intervet, Milan, Italy) and GNRH analog (Buserelin; Receptal. Hoechst-Roussel Vet, Milan, Italy). Protein concentration was determined by Bio-Rad Protein Assay Kit (Hercules, CA).

Animals and Hormonal Regimen

Sexually mature New Zealand white female rabbits (*Oryctolagus cuniculus*, 3.5–4 kg body weight, and 5 mo of age), were raised n premises owned by the University of Perugia and were used for all the experiments. The rabbits were housed individually in wire mesh cages under controlled light (14L:10D; lights off at 2100 h) and temperature (18°C–24°C) conditions. Each animal had free access to food and water. Pseudopregnancy was induced with 20 international units eCG followed 3 days later by 0.8 μg Buserelin [26]. The day of Buserelin injection was designated as Day 0. At Days 4 (early stage), 9 (midstage), and 13 (late stage) of pseudopregnancy, animals (n = 6 for each luteal stage) were euthanized by cervical dislocation in compliance with the guidelines of the Italian Ministry of Health (the national laws on animal research, DL 116/92) and the European Communities Council directive on animal research (N. 86/609/EEC). The protocols involving the use of the animals for these experiments were approved by the Bioethic Committee of the University of Perugia.

Tissue Collection and Processing

Upon euthanasia, the reproductive tracts were promptly removed and thoroughly washed with saline. Within a few minutes, all the CL were excised from ovaries (~12 CL/ovary), and after careful dissection of nonluteal tissue by fine forceps under stereoscopic magnification, some were immediately processed for in vitro experiments while others were rinsed with RNAse-free PBS and frozen at −80°C for later evaluation of gene expression. For the immunohistochemical detection of PPARγ, the ovaries of two additional animals for each luteal stage 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 [28].

Immunohistochemistry

Immunohistochemical investigation was performed according to procedures previously described [29, 30]. Slides were deparaffinized, rehydrated through graded concentrations of alcohol to distilled water, and submitted to antigen retrieval by microwaving in Tris solution (0.01 M, pH 8.5) at high power for 10 min. The slides were then cooled to room temperature. After rinsing with TBS (Tris-buffered saline), they were dipped in 3% H_2O_2 in methanol for 1 h to quench the endogenous peroxidase activity and rinsed in TBS (100 mM Tris, 75 mM NaCl; pH 7.4). Background labeling was prevented by incubating the sections with normal goat serum diluted 1:10 for 1 h at room temperature. The slides were then incubated overnight at 4°C in a moist chamber with the anti-PPARγ and anti-3β-HSD primary antibodies diluted 1:50 in TBS containing 0.2% Triton X-100 and 0.1% BSA. The next day, the slides were rinsed in TBS, treated again with normal goat serum and incubated with biotin goat anti-mouse secondary antibody diluted 1:200 for 30 min at room temperature. After TBS washes, the slides were exposed to the ABC kit for 30 min and rinsed again with TBS. The peroxidase activity sites were visualized using the DAB kit as the chromogen; the sections were rinsed with distilled water, washed in running tap water, dehydrated by passing through graded ethanol (70%, 95%, and 100% vol/vol) solutions, cleared in xylene, and, finally, mounted with Eukitt medium for light microscopy. Tissue sections in which the primary antibody was omitted or substituted by mouse IgG were used as negative controls of nonspecific staining. The intensity of immunostaining was assessed and compared microdensitometrically as previously described [31] using an image analysis system (IAAS 2000 image analyzer, Delta Sistemi, Rome, Italy).

RT-PCR

Total RNA was extracted from CL of three rabbits for each luteal stage as previously described [17]. Total RNA (5 μg) were reverse transcribed in 20 μl of SuperScript III Reverse Transcriptase cDNA synthesis mix using random hexamer according to the protocol provided by the manufacturer. Genomic DNA contamination was checked by developing the PCR without reverse transcriptase. Multiplex PCR amplification was carried out as previously described [17] using 1 μl of luteal cDNA as the template for the target (Table 1) and *18S* primers. Cycling conditions consisted of an initial denaturizing cycle at 94°C for 75 sec, followed by 35 cycles for each target gene at 94°C for 15 sec, 60°C for 30 sec, 72°C for 45 sec, 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. The amplified PCR-generated products (20 μl of 25 μl total reaction volume) were analyzed by electrophoresis on 2% agarose gel using ethidium bromide staining. Analysis of amplification products was carried out as reported elsewhere [17]. The amplified products, collected from agarose gel after electrophoresis, were purified with Nucleospin Extract II kit, and their identities were confirmed by DNA sequencing using the Sanger method.

In Vitro Experiments

A method previously reported was used for the in vitro study [32, 33]. Early, mid-, or late stage CL were randomly distributed into incubation wells in 1 ml of culture medium 199 with Earle Balanced Salt Solution containing 2.2 mg/ml sodium bicarbonate, 2.3mg/ml HEPES, and 3% BSA (w/v). Before the treatment, the CL were quartered inside each well (one quartered CL/well) using fine forceps. Each set of incubation wells was divided into three experimental groups, each formed of five wells as follows: (I) medium alone as control, (II) PPARγ agonist (15d-PGJ2, 200 nM), and (III) PPARγ antagonist (T0070907, 50 nM). The culture plates were incubated at 37°C in air with 5% CO_2. The media of each well were collected after 4 h of incubation and stored

TABLE 1. Primers for *PPARγ* and *18S* used as internal standard for RT-PCR quantification.

Gene	Product size	Primers (5′–3′)[a]
PPARγ	200 bp	F: TGAAGGATGCAAGGGTTTCT R: CCAACAGCTTCTCCTTCTCG
18S	489 bp	F: TCAAGAACGAAAGTCGGAGGTT R: GGACATCTAAGGGCATCA

[a] F, forward; R, reverse.

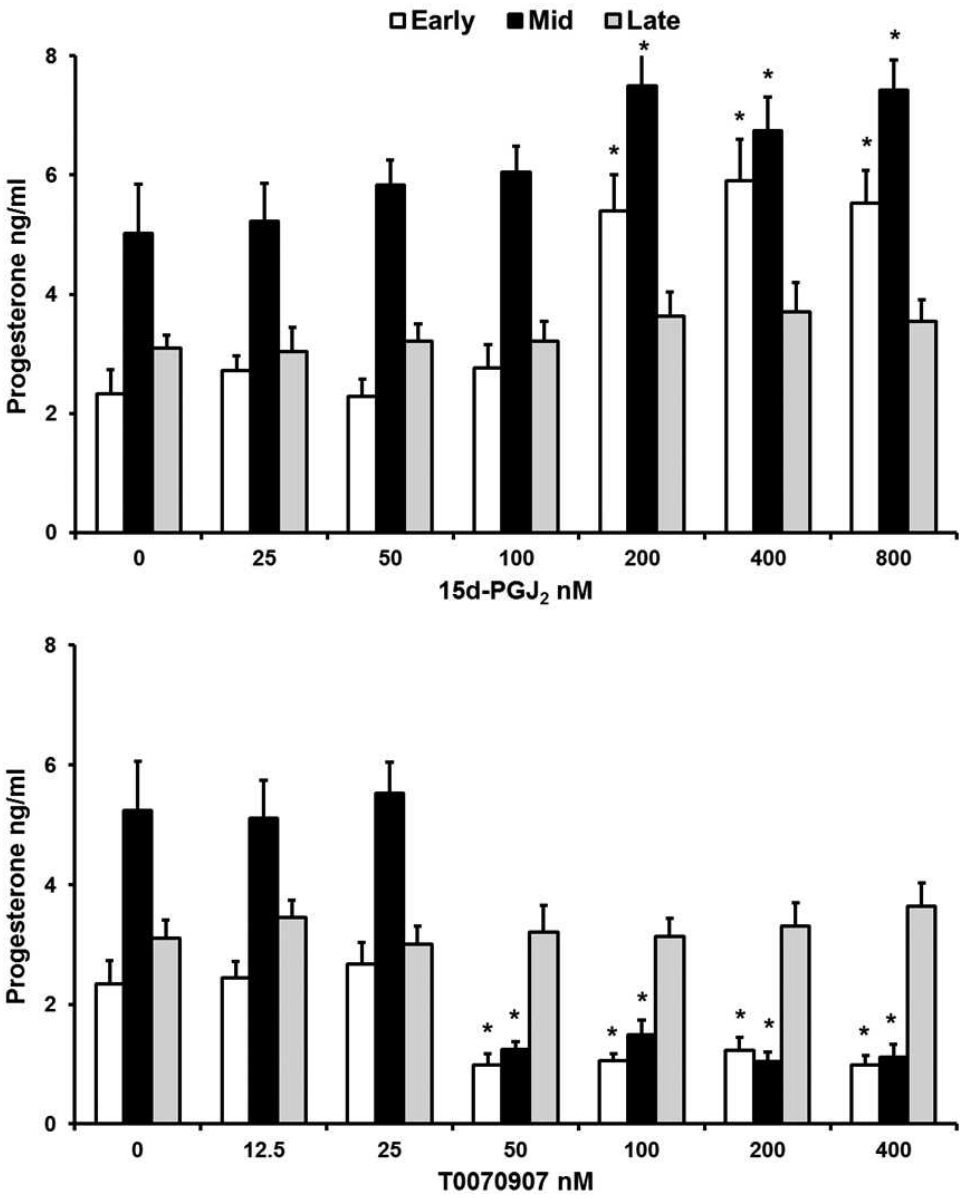

FIG. 1. In vitro effects of increasing concentrations of PPARγ agonist (15d-PGJ2) and antagonist (T0070907) on progesterone release by rabbit CL collected at early stage (Day 4), midstage (Day 9), and late stage (Day 13) of pseudopregnancy. Values are the means ± SD of six samples. Asterisks indicate a significantly different value ($P < 0.01$) versus control (0 nM).

immediately at −20°C for later determination of progesterone, PGF2α, and PGE2 well concentrations. Corpora lutea pieces were weighed and protein concentration was determined by an assay kit. Preliminary evidence led to our choice of the incubation conditions and the minimum effective dose for PPARγ agonist and antagonist used in the present in vitro study (Fig. 1).

Hormone Determination

Progesterone, PGF2α, and PGE2 concentrations were determined following the radioimmunoassay (RIA) protocols previously reported [32]. Intra- and interassay coefficients of variation and minimum detectable doses were: progesterone, 6.1%, 8.9%, and 11 pg/tube; PGF2α, 7.5%, 12.4%, and 18 pg/tube; and PGE2, 6.4%, 12.2%, and 16 pg/tube.

Enzyme Activity Determination

The activities of PTGS1 and PTGS2 were determined by measuring the disappearance of the radiolabeled substrate [^{3}H] AA using a method previously reported [21, 34]. A selective PTGS2 inhibitor (NS-398, 1 μM) and nonselective PTGS inhibitor (acetylsalicylic acid, 1 mM) were used. For each sample, PTGS1 activity was determined by calculating the rate of loss of [^{3}H] AA incubated with the selective PTGS2 inhibitor. Conversely, the PTGS2 activity of each corresponding sample was determined by calculating the rate of loss of [^{3}H] AA incubated without the selective PTGS2 inhibitor and subtracting from this the value of the PTGS1 activity. The values for PTGS1 and PTGS2 were corrected by subtracting [^{3}H] AA disappearance resulting from other enzymatic activities and nonenzymatic reactions. The activity of PGE2-9-K was determined by measuring the conversion of [^{3}H] PGE2 into [^{3}H] PGF2α using a previously reported modified method [21].

The activity of 3β-HSD was determined by measuring the conversion of [^{3}H] pregnenolone into [^{3}H] progesterone. Briefly, each pool of CL was homogenized in 1 ml cold fresh homogenizing buffer (20 mM K_2HPO_4, 1 mM ethylenediaminetetraacetic acid [EDTA] and 10 mM β-mercaptoethanol, pH 7.4). The total homogenate was filtered and immediately used for the assay of enzymatic activity. One hundred microliters of homogenate and 50 μl of homogenizing buffer containing 150 000 decompositions/min (d.p.m.) [^{3}H] pregnenolone 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 the addition of 100 μl 0.1 M HCl. Steroids were extracted with diethyl ether and resuspended with 500 ml RIA buffer (74.5 mM Na_2HPO_4, 12.5 mM sodium EDTA, 0.1% gelatin, pH 7.5). Two hundred microliters RIA buffer containing progesterone-specific antiserum were added to duplicate samples, and the

TABLE 2. Two-way ANOVA *F* values: treatments (control, 15d-PGJ2, T0070907) versus CL stages (early, mid, late).[a]

Parameter	Treatments	CL stages	Interaction
Progesterone	17.12*	26.23*	1.24
PGF2α	26.09*	18.23*	2.31
PGE2	3.12	33.62*	0.89
PTGS1	2.58	15.99*	1.32
PTGS2	37.15*	26.04*	2.46
PGE2-9-K	3.51	16.27*	2.74
3β-HSD	24.39*	18.92*	2.38
20α-HSD	2.65	10.52*	1.16

[a] Degrees of freedom: treatments, 2; CL stages, 2; interaction, 4; error, 45; *$P < 0.01$.

mixture was incubated at 4°C for 16 h. The [^{3}H] progesterone-antiserum bound fraction was separated with charcoal-dextran suspension, and the radioactivity was quantified by liquid scintillation counting. The sample and substrate mixture with a specific 3β-HSD inhibitor (trilostane, 1 mM) was used to determine the [^{3}H] progesterone synthesis resulting from other enzymatic activities and nonenzymatic reactions. The values for 3β-HSD were corrected by subtracting the values due to other enzymatic activities and nonenzymatic reactions.

The activity of 20α-HSD was determined by measuring the residual level of [^{3}H] progesterone in a homogenate of incubated CL. Briefly, 100 μl of CL homogenate and 50 μl of homogenizing buffer containing 150 000 d.p.m. [^{3}H] progesterone were added to the incubation tube. The mixture was incubated at 37°C for 10 min. Termination was achieved by the addition of 100 μl 0.1 M HCl. The [^{3}H] progesterone-antiserum bound fraction was quantified as reported above for 3β-HSD. The sample and substrate mixture with a specific 20α-HSD inhibitor (R-flurbiprofen, 1 mM) was used to determine the [^{3}H] progesterone disappearance resulting from other enzymatic activities and nonenzymatic reactions. The values for 20α-HSD were corrected by subtracting the values due to other enzymatic activities and nonenzymatic reactions.

Statistical Analysis

All the data were examined by Levene test, which showed statistically significant homoscedasticity for all of the variables. Data were analyzed using two-way ANOVA (treatments vs. CL stages, Table 2) followed by Student-Newman-Keuls *t*-test. Differences were considered significant at $P < 0.01$.

RESULTS

Immunohistochemistry

Intense PPARγ immunoreactivity was localized in the perinuclear cytoplasm and, with lesser intensity, in the nucleus of all the luteal cells at the early stage of pseudopregnancy (Fig. 2, A and B). The density of immune reactive cells decreased ($P < 0.01$) from the early to late stage (Fig. 2, C, D, and G); in particular, the majority of the luteal cell nuclei were immunonegative at the late stage (Fig. 2D). The wall of blood vessels (endothelial, stromal, and smooth muscle cells) was always negative (Fig. 2E). No staining was detectable in negative control rabbit CL in which the primary antibody was omitted or substituted with mouse IgG (Fig. 2F).

Expression of mRNA for Luteal PPARγ

PPARγ transcript was expressed in CL at different stages of pseudopregnancy with the lowest abundance ($P < 0.01$) at the late stage of pseudopregnancy (Fig. 3, upper panel).

In Vitro Hormone Production

Basal progesterone production of midstage CL was higher ($P < 0.01$) than that of both early and late stages CL, and secretion by late stage CL was higher ($P < 0.01$) than that of the early ones (Fig. 4, upper panel). The PPARγ agonist 15d-PGJ2 increased ($P < 0.01$) and the antagonist T0070907 reduced ($P < 0.01$) in vitro progesterone secretion in early and midluteal stages of pseudopregnancy (Fig. 4, upper panel).

Basal PGF2α secretion was higher in late CL ($P < 0.01$) than in the early and midluteal stages, and secretion by midstage CL was higher ($P < 0.01$) than that of early ones (Fig. 4, middle panel). The PPARγ agonist 15d-PGJ2 reduced ($P < 0.01$) and the antagonist T0070907 increased ($P < 0.01$) PGF2α secretion in early and midluteal stages of pseudopregnancy (Fig. 4, middle panel).

Basal PGE2 secretion was higher in early CL stage ($P < 0.01$) than in midluteal and late stages, and secretion by late CL stage was higher ($P < 0.01$) than that of midstage CL (Fig. 4, lower panel). Both 15d-PGJ2 and T0070907 did not modify PGE2 in vitro secretion in any luteal stage (Fig. 4, lower panel).

Luteal Enzyme Activities

Basal PTGS1 activity was higher in the late CL stage ($P < 0.01$) than in early and midstages (Fig. 5, upper panel), whereas PTGS2 activity significantly increased ($P < 0.01$) with the progress of the CL stages (Fig. 5, middle panel). Both 15d-PGJ2 and T0070907 did not affect PTGS1 activity in any CL types (Fig. 5, upper panel), while 15d-PGJ2 reduced ($P < 0.01$) PTGS2 activity at early stage of pseudopregnancy and T00070907 increased it ($P < 0.01$) at the midstage (Fig. 5, middle panel).

Basal PGE2-9-K activity of late stage CL was higher ($P < 0.01$) than that of early and midstages, and the activity of midstage CL was higher ($P < 0.01$) than that of the early ones (Fig. 5, lower panel). Both 15d-PGJ2 and T0070907 did not affect PGE2-9-K activity in any CL type (Fig. 5, lower panel).

Basal 3β-HSD activity was higher in midstage CL ($P < 0.01$) than in early and late stage CL, and activity in late stage CL was lower ($P < 0.01$) than that of early ones (Fig 6, upper panel). The agonist 15d-PGJ2 increased ($P < 0.01$) and the antagonist T0070907 reduced ($P < 0.01$) 3β-HSD activity at early and midstages of pseudopregnancy (Fig. 6, upper panel).

Basal 20α-HSD activity was lower in midstage CL ($P < 0.01$) than in early and late stage CL, and the activity of late stage CL was higher ($P < 0.01$) than that of early ones (Fig. 6, lower panel). Both 15d-PGJ2 and T0070907 did not affect 20α-HSD activity in any CL types (Fig. 6, lower panel).

DISCUSSION

The present study provides evidence for the first time of the presence of PPARγ in rabbit CL. The results indicate that this nuclear transcription factor may well be another likely candidate for the list of factors known to be involved in luteotropic function.

Several studies have reported the occurrence of PPARγ in mammalian ovary; this nuclear receptor has been found in the granulosa and theca cells and also in CL of rodents and ruminants [7, 35, 36]. In addition, PPARγ was detected in primary and secondary follicles, being more expressed in the large follicles and decreasing with the LH surge [2, 7, 36]. In bovine CL, *PPARγ* gene and protein expressions increased after ovulation, but decreased if no fertilization or embryo implantation occurred [37, 38].

Our immunohistochemical results demonstrated the presence of PPARγ in the cytoplasm and nuclei of all luteal cells during the three stages of pseudopregnancy examined. Of note, in late stage CL, the majority of nuclei were immunonegative. This finding was partly supported by the results on gene expression; indeed, PCR showed the lowest amounts of *PPARγ*

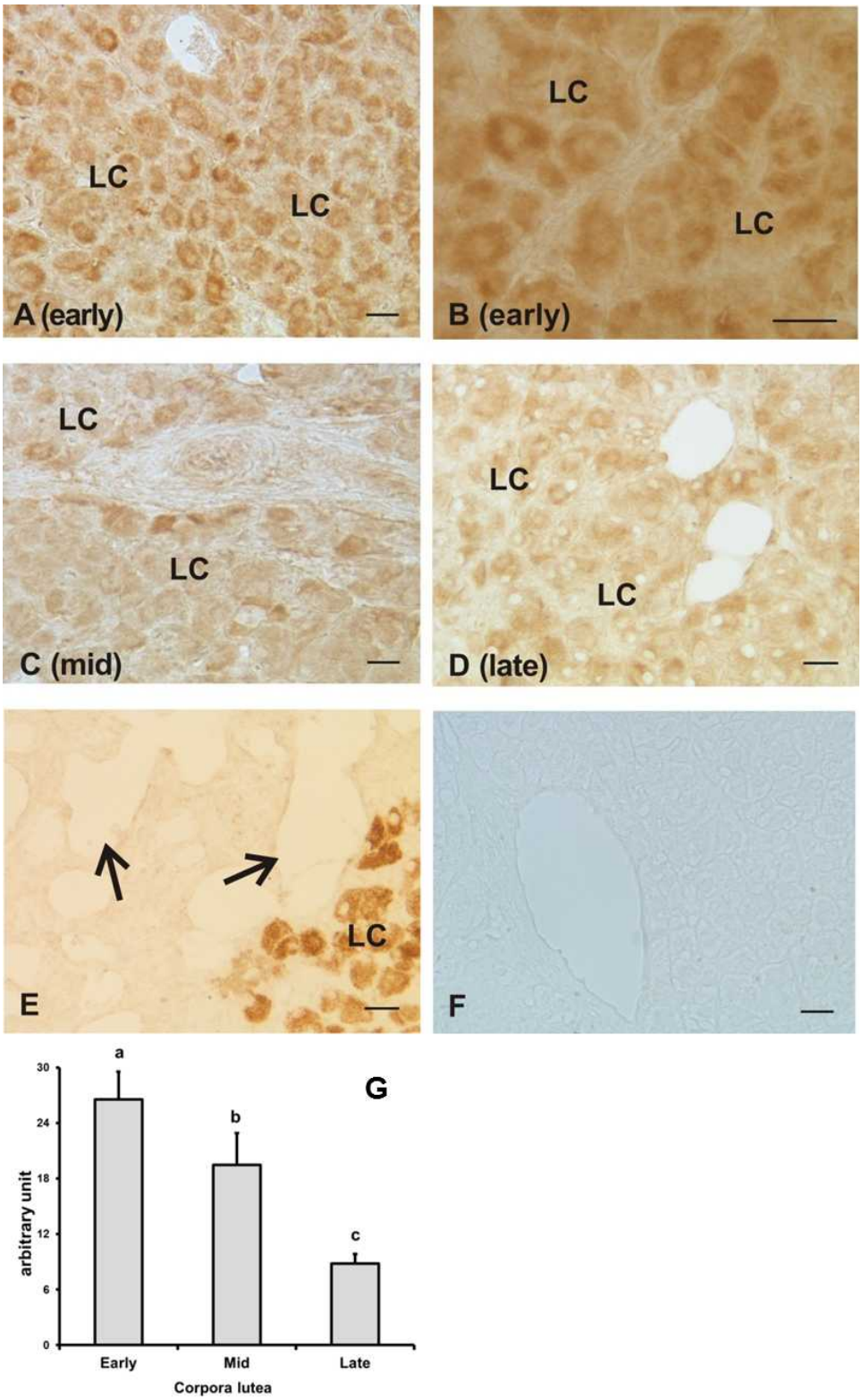

FIG. 2. Immunohistochemical detection of PPARγ in rabbit CL collected at early stage (Day 4), midstage (Day 9), and late stage (Day 13) of pseudopregnancy. **A**, **B**) Early CL: the immunosignals are intensely localized in the perinuclear cytoplasm and nucleus of luteal cells (LC) with **B** at a higher magnification of **A**. **C**) Midstage CL: a decrease of the immunosignals in luteal cells is evident. **D**) Late stage CL: the majority of the luteal cell nuclei are immunonegative. **E**) Early stage CL: the wall of the blood vessels (arrows) are immunonegative. **F**) Early stage CL: section of negative control. Bars = 20 μm. **G**) Immunodensitometry: data are expressed in arbitrary units; values for each luteal stage are the means ± SD of 20 slides; and different letters above the bars indicate significantly different values ($P < 0.01$).

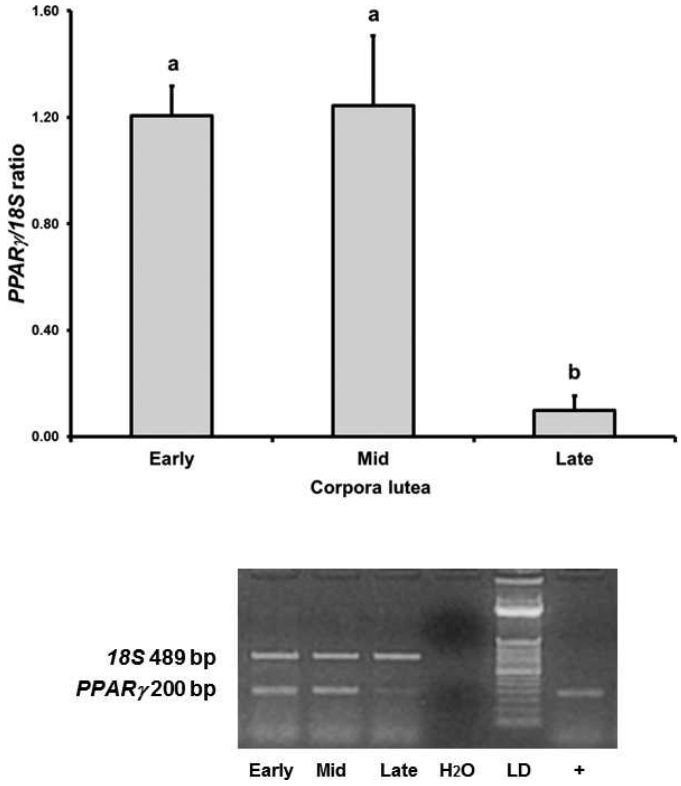

FIG. 3. Expression profile of *PPARγ* mRNA in rabbit CL collected at early stage (Day 4), midstage (Day 9), and late stage (Day 13) of pseudopregnancy. The lower panel shows a representative photograph of a 2% agarose ethidium bromide-stained gel used to analyze the PCR products. The sizes of the amplified products are shown on the left of the gel. Lane LD is the kilobase DNA marker, lane H_2O represents a water control (substitution of water for the template), and lane + is a positive control (rabbit kidney for the template). The other lanes identify the corresponding stages of pseudopregnancy. The upper panel shows the data derived from densitometric analyses of the gels. For each luteal stage, the values (means ± SD) combine the results from three different rabbits and are reported in arbitrary units of *PPARγ* mRNA relative to that of *18S* used as an internal standard. Different letters above the bars indicate significantly different values ($P < 0.01$).

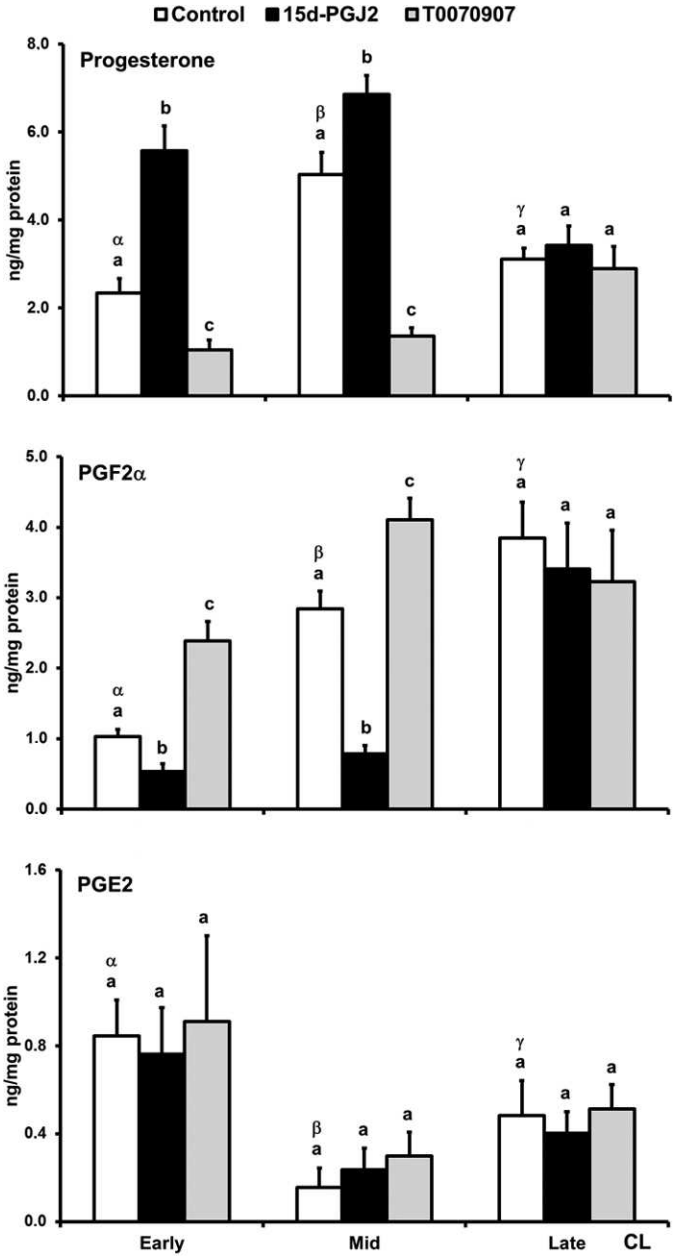

FIG 4. In vitro effects of PPARγ agonist (15d-PGJ2) and antagonist (T0070907) on progesterone, PGF2α, and PGE2 releases by rabbit CL collected at early stage (Day 4), midstage (Day 9), and late stage (Day 13) of pseudopregnancy. Values are the means ± SD of six samples. Different letters above the bars indicate significantly different values ($P < 0.01$): Greek letters among the control CL stages; Latin letters among the experimental groups of the same CL stages.

mRNA in late CL. Even if the protein and gene presence did not necessarily imply a role, results indicate that PPARγ has a role in regulating the CL life span. In this context, PPARγ tissue-specific deletion in mouse ovary led to a decrease in fertility [5], and Froment et al. [1] found that PPARγ plays a significant role in fertility control, preserving CL function during pregnancy.

Komar [2] reported that PPARγ activation affects progesterone production by ovarian cells. In particular, 15d-PGJ2, an endogenous activator of PPARγ, inhibited the basal and gonadotropin-stimulated progesterone production in human granulosa cells [39], whereas 15d-PGJ2 and ciglitazone, a synthetic PPARγ activator, increased the granulosa cell progesterone secretion in eCG-primed immature rats [36]. Porcine theca and bovine luteal cells also showed an increase of progesterone production after PPARγ activation by 15d-PGJ2, ciglitazone, or troglitazone, another synthetic activator, [6, 37]. These studies clearly demonstrate that the effect of PPARγ on progesterone synthesis depends on cell type, stage of cell differentiation, stage of the ovarian cycle, and/or animal species [2].

The present research showed that 15d-PGJ2 induced an increase of progesterone production in early and midluteal stage CL cultured in vitro, whereas the inhibitor T0070907 displayed the opposite effect. In contrast, late stage CL were not affected by 15d-PGJ2 or by T0070907 in vitro treatments. The lack of effects by PPARγ activator and inhibitor in late stage CL may depend on the disappearance of PPARγ in the nucleus of most luteal cells at this stage of pseudopregnancy, as indirectly demonstrated by immunohistochemical and gene expression results.

Various studies used thiazolidinediones (TZDs), a class of PPARγ activators employed as drugs in diabetes mellitus, to evaluate the effects of this nuclear receptor on the synthesis of ovarian steroid hormones and the expression of many rate-

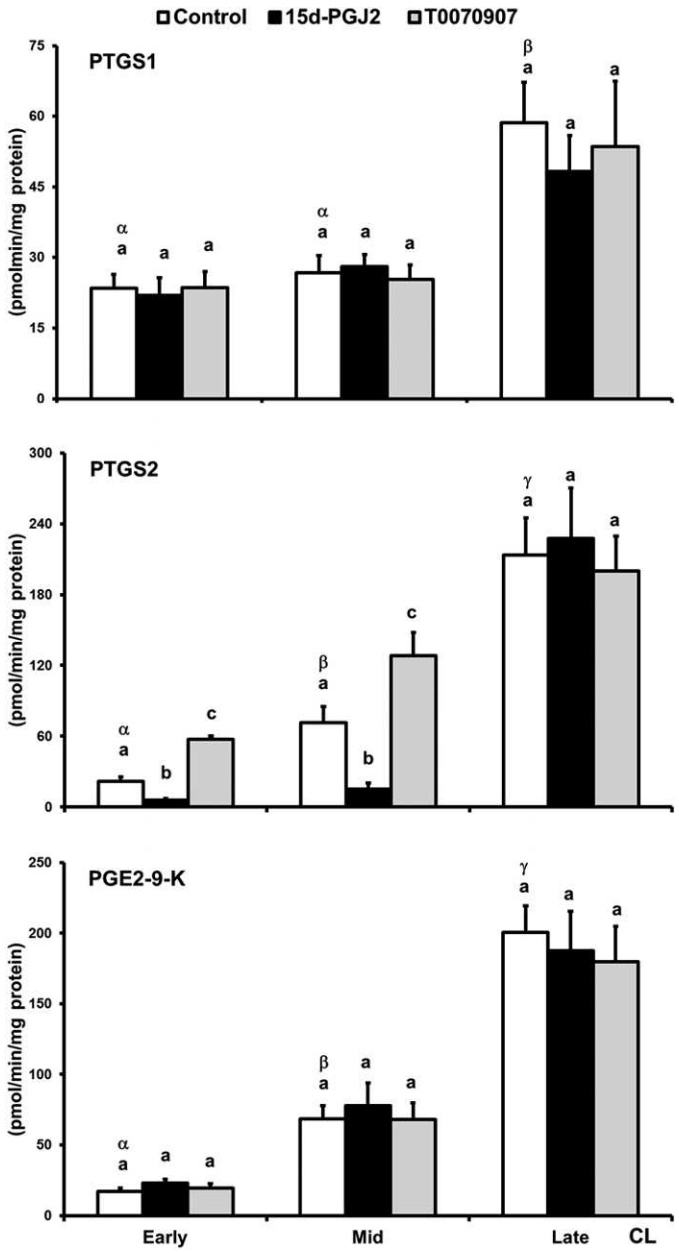

FIG. 5. In vitro effects of PPARγ agonist (15d-PGJ2) and antagonist (T0070907) on PTGS1, PTGS2, and PGE2-9-K activities of rabbit CL collected at early stage (Day 4), midstage (Day 9), and late stage (day 13) of pseudopregnancy. The enzyme activities were linear with time and amount of protein. Substrate conversion: PTGS1, early 18%, mid 21%, late 44%; PTGS2, early 7%, mid 52%, late 8%; PGE2-9-K, early 4%, mid 16%, late 46%. Values are the means ± SD of six samples. Different letters above the bars indicate significantly different values ($P < 0.01$): Greek letters among the control CL stages; Latin letters among the experimental groups of the same CL stages.

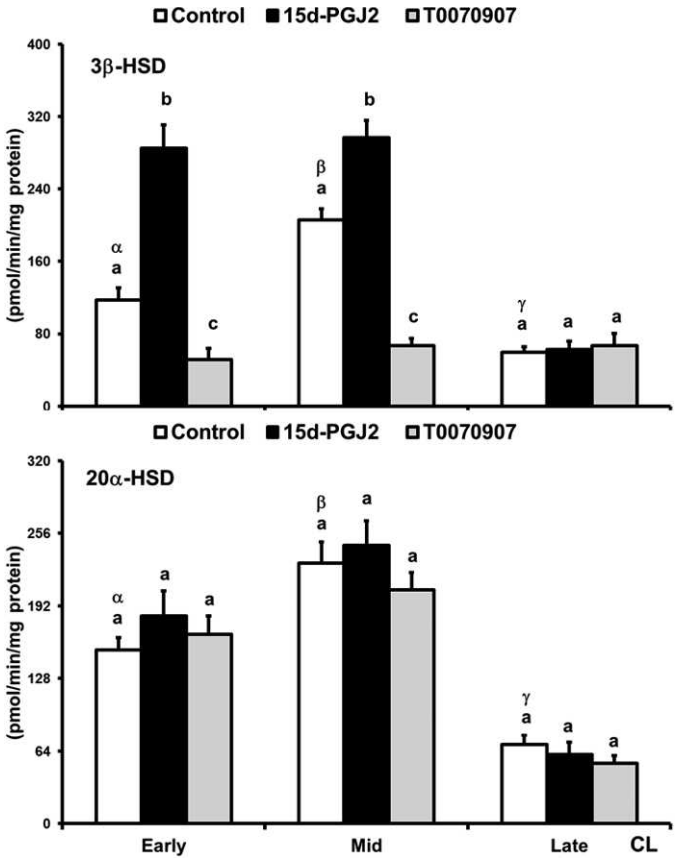

FIG. 6. In vitro effects of PPARγ agonist (15d-PGJ2) and antagonist (T0070907) on 3β-HSD and 20α-HSD activities of rabbit CL collected at early stage (Day 4), midstage (Day 9), and late stage (Day 13) of pseudopregnancy. The enzyme activities were linear with time and amount of protein. Substrate conversion: 3β-HSD, early 19%, mid 33%, late 10%; 20α-HSD, early 18%, mid 8%, late 27%. Values are the means ± SD of six samples. Different letters above the bars indicate significantly different values ($P < 0.01$): Greek letters among the control CL stages; Latin letters among the experimental groups of the same CL stages.

limiting steroidogenic enzymes [3]. In particular, 3β-HSD activity was inhibited by troglitazone in porcine granulosa cells [40], whereas, other authors argued that TZD treatment did not affect *3β-HSD* mRNA levels in porcine granulosa cells and protein concentrations in ovine granulosa cells [41, 42].

In contrast, our data demonstrated that PPARγ affects the 3β-HSD activity because 15d-PGJ2, increased and T007090 diminished the activity of this steroidogenic enzyme in early and midstages CL. It is interesting to note that neither the activator nor the inhibitor of PPARγ had any effects on 3β-HSD activity during the late luteal stage. Furthermore, 20α-HSD enzymatic activity was not modified by 15d-PGJ2 or by T0070907 in any luteal stage.

The molecular mechanisms of PPARγ in ovarian functions are not well understood, notwithstanding the suggestion that PPARγ can directly influence *PTGS2* gene expression [1]. In fact, the *PTGS2* promoter region contains a PPARγ response element [43]. On the other hand, there are conflicting studies that report both PPARγ stimulating [40] and inhibiting [44, 45] effects on *PTGS2* gene expression.

The present study found evidence for PPARγ down-regulation of PTGS2 luteal activity at both the early and midstages of pseudopregnancy, as suggested by the coherent effects induced by 15d-PGJ2 and the opposite ones by T0070907. Our data indirectly indicate that this PTGS2 down-regulation may cause a decrease of PGF2α in vitro secretion by early and midstages CL, whereas neither PTGS1 activity nor PGE2 release were modified. In rabbit CL, PGE2-9-K has a modulator role that correlates its enzymatic activity with PGE2 and PGF2α synthesis negatively and positively, respectively [21]. Wintergalen et al. [46] found that in the rabbit, luteal PGE2-9-K also exerts an effect on 20α-HSD catalytic activity, thus favoring the conversion of progesterone into the inactive metabolite 20α-OH-progesterone, with a clear luteolytic effect. Interestingly, these results for 20α-HSD and

PGE2-9-K activity fit with similar findings in the endometrium [47] and placentomes [48] of bovine. As we found for 20α-HSD, PGE2-9-K activity was not modified by 15d-PGJ2 or T0070907 treatments in any luteal stage.

In conclusion, our research found evidence for a possible luteotropic role for PPARγ in the pseudopregnant rabbit, with a mechanism that involves the up-regulation of 3β-HSD and the increase of progesterone as well as the down-regulation of the luteolytic factor PGF2α and its correlated enzyme PTGS2 [21]. This hypothetical mechanism is further supported by the clear reduction of the presence of PPARγ in the nucleus of luteal cells in late stage4 CL, a reduction that could be required for luteolysis. However, although the present data shed new light on possible PPARγ physiological mechanisms regulating luteal activity of mammals, further studies are needed to better understand the fine-tuning of this nuclear receptor in controlling the luteal life span.

ACKNOWLEDGMENT

The authors gratefully acknowledge the revision of the English text by Sheila Beatty.

REFERENCES

1. Froment P, Gizard F, Defever D, Staels B, Dupont J, Monget P. Peroxisome proliferator-activated receptors in reproductive tissues: from gametogenesis to parturition. J Endocrinol 2006; 189:199–209.
2. Komar CM. Peroxisome proliferator-activated receptors (PPARs) and ovarian function—implications for regulating steroidogenesis, differentiation, and tissue remodelling. Reprod Biol Endocrinol 2005; 3:41.
3. Minge CE, Robker RL, Norman RJ. PPAR gamma: coordinating metabolic and immune contributions to female fertility. PPAR Res 2008; 2008:243791.
4. Braissant O, Foufelle F, Scotto C, Dauça M, Wahli W. Differential expression of peroxisome proliferator-activated receptors (PPARs): tissue distribution of PPAR-α, -β, and -γ in the adult rat. Endocrinology 1996; 137:354–366.
5. Cui Y, Miyoshi K, Claudio E, Siebenlist UK, Gonzalez FJ, Flaws J, Wagner KU, Hennighausen L. Loss of the peroxisome proliferation-activated receptor γ (PPARγ) does not affect mammary development and propensity for tumor formation but leads to reduced fertility. J Biol Chem 2002; 277:17830–17835.
6. Schoppee PD, Garmey JC, Veldhuis JD. Putative activation of the peroxisome proliferator-activated receptor γ impairs androgen and enhances progesterone biosynthesis in primary cultures of porcine theca cells. Biol Reprod 2002; 66:190–198.
7. Froment P, Fabre S, Dupont J, Pisselet C, Chesneau D, Staels B, Monget P. Expression and functional role of peroxisome proliferator-activated receptor-γ in ovarian folliculogenesis in the sheep. Biol Reprod 2003; 69: 1665–1674.
8. Löhrke B, Viergutz T, Shahi SK, Pöhland R, Wollenhaupt K, Goldammer T, Walzel H, Kanitz W. Detection and functional characterisation of the transcription factor peroxisome proliferator-activated receptor γ in lutein cells. J Endocrinol 1998; 159:429–439.
9. Sundvold H, Brzozowska A, Lien S. Characterisation of bovine peroxisome proliferator-activated receptors γ1 and γ2: genetic mapping and differential expression of the two isoforms. Biochem Biophys Res Comm 1997; 239:857–861.
10. Parillo F, Catone G, Gobbetti A, Zerani M. Cell localization of ACTH, dopamine, and GnRH receptors and PPARγ in bovine corpora lutea during diestrus. Acta Sci Vet 2013; 41:e1129.
11. Maranesi M, Zerani M, Parillo F, Brecchia G, Gobbetti A, Boiti C. Role of peroxisome proliferator-activated receptor gamma in corpora lutea of pseudopregnant rabbits. In: Abstracts of 15th Annual Conference of the European-Society-for-Domestic-Animal-Reproduction (ESDAR), September 15–17, 2011, Antalya, Turkey. Reprod Domest Anim 2011; 46(Supplement 3):26.
12. Lambe KG, Tugwood JD. A human peroxisome-proliferator-activated receptor-γ is activated by inducers of adipogenesis, including thiazalidinedione drugs. Eur J Biochem 1996; 239:1–7.
13. Mu YM, Yanase T, Nishi Y, Waseda N, Oda T, Tanaka A, Takayanagi R, Nawata H. Insulin sensitizer, troglitazone, directly inhibits aromatase activity in human ovarian granulosa cells. Biochem Biophys Res Comm 2000; 271:710–713.
14. Niswender GD, Juengel JL, Silva PJ, Rollyson MK, McIntush EW. Mechanisms controlling the function and life span of the corpus luteum. Physiol Rev 2000; 80:1–29.
15. Davis JS, Rueda BR. The corpus luteum: an ovarian structure with maternal instincts and suicidal tendencies. Front Biosci 2002; 7: d1949–d1978.
16. Wiltbank MC, Ottobre JS. Regulation of intraluteal production of prostaglandins. Reprod Biol Endocrinol 2003; 1:91.
17. Boiti C, Guelfi G, Brecchia G, Dall'Aglio C, Ceccarelli P, Maranesi M, Mariottini C, Zampini D, Gobbetti A, Zerani M. Role of endothelin-1 system in the luteolytic process of pseudopregnant rabbits. Endocrinology 2005; 146:1293–1300.
18. Helliwell RJA, Adams LF, Mitchell MD. Prostaglandin synthases: recent developments and a novel hypothesis. Prostaglandins Leukot Essent Fatty Acids 2004; 70:101–113.
19. Boiti C, Zerani M, Zampini D, Gobbetti A. Nitric oxide synthase activity and progesterone release by isolated corpora lutea of rabbits in early and mid-luteal phase of pseudopregnancy are differently modulated by prostaglandin E2 and prostaglandin F2α via adenylate cyclase and phospholipase C. J Endocrinol 2000; 164:179–186.
20. Arosh JA, Banu SK, Chapdelaine P, Madore E, Sirois J, Fortier MA. Prostaglandin biosynthesis, transport and signaling in corpus luteum: a basis for autoregulation of luteal function. Endocrinology 2004; 145: 2551–2560.
21. Zerani M, Dall'Aglio C, Maranesi M, Gobbetti A, Brecchia G, Mercati F, Boiti C. Intraluteal regulation of prostaglandin F2alpha-induced prostaglandin biosynthesis in pseudopregnant rabbits. Reproduction 2007; 133: 1005–1016.
22. Zerani M, Catone G, Maranesi M, Gobbetti A, Boiti C, Parillo F. Gonadotropin-releasing hormone 1 directly affects corpora lutea life-span in Mediterranean buffalo (*Bubalus bubalis*) during diestrus: presence and in vitro effects on enzymatic and hormonal activities. Biol Reprod 2012; 87:1–8.
23. Mendez M, LaPointe MC. PPARgamma inhibition of cyclooxygenase-2, PGE2 synthase, and inducible nitric oxide synthase in cardiac myocytes. Hypertension 2003; 42:844–850.
24. Forman BM, Tontonoz P, Chen J, Brun RP, Spiegelman BM, Evans RM. 15-deoxy-Δ12, 14-prostaglandin J2 is a ligand for the adipocyte determination factor PPARγ. Cell 1995; 83:803–812.
25. Bishop-Bailey D, Wray J. Peroxisome proliferator-activated receptors: a critical review on endogenous pathways for ligand generation. Prostaglandins Other Lipid Mediat 2003; 71:1–22.
26. Stradaioli G, Verini Supplizi A, Monaci M, Canali C, Boiti C. Effects of different doses of PMSG on ovarian response and in vitro embryo development in rabbit. World Rabbit Sci 1997; 5:143–148.
27. Lee G, Elwood F, McNally J, Weiszmann J, Lindstrom M, Amaral K, Nakamura M, Miao S, Cao P, Learned RM, Chen JL, Li Y. T0070907, a selective ligand for peroxisome proliferator-activated receptor gamma, functions as an antagonist of biochemical and cellular activities. J Biol Chem 2002; 277:19649–19657.
28. Parillo F, Catone G, Maranesi M. Immunolocalization, gene expression, and enzymatic activity of cyclooxygenases, prostaglandin e2-9-ketoreductase, and nitric oxide synthases in Mediterranean buffalo *(Bubalus bubalis*) corpora lutea during diestrus. Microsc Res Tech 2012; 75: 1682–1690.
29. Parillo F, Catone G, Boiti C, Zerani M. Immunopresence and enzymatic activity of nitric oxide synthases, cyclooxygenases and PGE2-9-ketoreductase and in vitro production of PGF2α, PGE2 and testosterone in the testis of adult and prepubertal alpaca (*Lama pacos*). Gen Comp Endocrinol 2011; 171:381–388.
30. Zerani M, Catone G, Quassinti L, Maccari E, Bramucci M, Gobbetti A, Maranesi M, Boiti C, Parillo F. In vitro effects of GnRH on Leydig cells of adult alpaca (*Lama pacos*) testis: GnRH receptor immunolocalization, testosterone and prostaglandin synthesis, and cyclooxygenase activities. Dom Anim Endocrinol 2011; 40:51–59.
31. Zerani M, Parillo F, Brecchia G, Guelfi G, Dall'Aglio C, Lilli L, Maranesi M, Gobbetti A, Boiti C. Expression of type I GNRH receptor and in vivo and in vitro GNRH-I effects in corpora lutea of pseudopregnant rabbits. J Endocrinol 2010; 207:289–300.
32. Boiti C, Zampini D, Zerani M, Guelfi G, Gobbetti A. Prostaglandin receptors and role of G protein-activated pathways on corpora lutea of pseudopregnant rabbit in vitro. J Endocrinol 2001; 168:141–151.
33. Parillo F, Dall'Aglio C, Brecchia G, Maranesi M, Polisca A, Boiti C, Zerani M. Aglepristone (RU534) effects on luteal function of pseudo-

pregnant rabbits: steroid receptors, enzymatic activities, and hormone productions in corpus luteum and uterus. Anim Reprod Sci 2013; In press.
34. Zerani M, Catone G, Betti G, Parillo F. Immunopresence and functional activity of prostaglandin-endoperoxide synthases and nitric oxide synthases in bovine corpora lutea during diestrus. Folia Morphol 2013: In press.
35. Gasic S, Bodenburg Y, Nagamani M, Green A, Urban RJ. Troglitazone inhibits progesterone production in porcine granulosa cells. Endocrinology 1998; 139:4962–4966.
36. Komar CM, Braissant O, Wahli W, Curry TE Jr. Expression and localization of PPARs in the rat ovary during follicular development and the periovulatory period. Endocrinology 2001; 142:4831–4838.
37. Lšhrke B, Viergutz T, Shahi SK, Pöhland R, Wollenhaupt K, Goldammer T, Walzel H, Kanitz W. Detection and functional characterisation of the transcription factor peroxisome proliferator-activated receptor gamma in lutein cells. J Endocrinol 1998; 159:429–439.
38. Viergutz T, Lšhrke B, Poehland R, Becker F, Kanitz W. Relationship between different stages of the corpus luteum and the expression of the peroxisome proliferator-activated receptor gamma protein in bovine large lutein cells. J Reprod Fertil 2000; 118:153–161.
39. Willis DS, White J, Brosens S, Franks S. Effect of 15-deoxy-delta (12,14)-prostaglandin J2 (PGJ2) a peroxisome proliferator activating receptor γ (PPARγ) ligand on human ovarian steroidogenesis. Endocrinology 1999; 140(suppl 1):491.
40. Gasic S, Nagamani M, Green A, Urban RJ. Troglitazone is a competitive inhibitor of 3 beta-hydroxysteroid dehydrogenase enzyme in the ovary. Am J Obst Gynecol 2001; 184:575–579.
41. Doney A, Fischer B, Frew D, Cumming A, Flavell DM, World M, Montgomery HE, Boyle D, Morris A, Palmer CN. Haplotype analysis of the PPARγ Pro12Ala and C1431T variants reveals opposing associations with body weight. BMC Genetics 2002; 3:21.
42. Ek J, Andersen G, Urhammer SA, Hansen L, Carstensen B, Borch-Johnsen K, Drivsholm T, Berglund L, Hansen T, Lithell H, Pedersen O. Studies of the Pro12Ala polymorphism of the peroxisome proliferator-activated receptor-γ2 (PPAR-γ2) gene in relation to insulin sensitivity among glucose tolerant caucasians. Diabetologia 2001; 44:1170–1176.
43. Meade E, McIntyre T, Zimmerman G, Prescott S. Peroxisome proliferators enhance cyclooxygenase-2 expression in epithelial cells. J Biol Chem 1999; 274:8328–8334.
44. Subbaramaiah K, Liu DT, Hart JC, Dannenberg AJ. Peroxisome proliferator-activated receptor γ ligands suppress the transcriptional activation of cyclooxygenase-2. J Biol Chem 2001; 276:12440–12448.
45. Inoue H, Tanabe T, Umesono K. Feedback control of cyclooxygenase-2 expression through PPARγ. J Biol Chem 2000; 275:28028–28032.
46. Wintergalen N, Thole HH, Galla HJ, Schlegel W. Prostaglandin-E2 9-reductase from corpus luteum of pseudopregnant rabbit is a member of the aldo-keto reductase superfamily featuring 20alpha-hydroxysteroid dehydrogenase activity. Eur J Biochem 1995; 15:264–270.
47. Madore E, Harvey N, Parent J, Chapdelaine P, Arosh JA, Fortier MA. An aldose reductase with 20 alpha-hydroxysteroid dehydrogenase activity is most likely the enzyme responsible for the production of prostaglandin F2 alpha in the bovine endometrium. J Biol Chem 2003; 278:11205–11212.
48. Schuler G, Teichmann U, Kowalewski MP, Hoffmann B, Madore E, Fortier MA, Klisch K. Expression of cyclooxygenase-II (COX-II) and 20 alpha-hydroxysteroid dehydrogenase (20 alpha-HSD)/prostaglandin F-synthase (PGFS) in bovine placentomes: implications for the initiation of parturition in cattle. Placenta 2006; 27:1022–1029.