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# Vasoactive Peptides in the Luteolytic Process Activated by PGF2alpha in Pseudopregnant Rabbits at Different Luteal Stages[1]

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## ABSTRACT

**To study the role of endothelial factors in luteal function, the dynamic profiles of genes for endothelin 1 (*EDN1*), its receptor subtypes, *EDNRA* and *EDNRB*, and angiotensin converting enzyme (*ACE*) were examined in corpora lutea (CL) obtained from rabbits on Days 4 and 9 of pseudopregnancy after prostaglandin (PG) F2α analogue (alfaprostol) treatment. The cell type distribution of EDN1 in the ovaries and its mechanisms of actions in vitro and in vivo were also studied. Positive immunostaining for EDN1 was localized in the luteal and endothelial cells, in granulosa cells of the follicles, and in the ovarian epithelium. The basal mRNA levels for *EDNRA*, *EDNRB*, and *ACE* were lower ($P \leq 0.01$) in Day-4 CL than in Day-9 CL, whereas those for *EDN1* did not differ between these two time-points. On Day 4, the luteal *EDN1*, *EDNRA*, *EDNRB*, and *ACE* mRNA levels were similarly increased two-fold ($P \leq 0.01$) 1.5 h after alfaprostol injection, and did not show further changes in the subsequent 24 h. On Day 9, alfaprostol challenge transiently up-regulated ($P \leq 0.01$) the luteal *ACE* transcripts at 1.5 h, and those of *EDN1* at 1.5 h and 3 h, whereas the *EDNRA* and *EDNRB* transcript levels remained unchanged during the course of luteal regression. EDN1 decreased ($P \leq 0.01$) progesterone release and increased ($P \leq 0.01$) PGF2α secretion and NOS activity via the PLC/PKC pathway in Day-9 CL, but not in Day-4 CL, cultured in vitro. EDN1-induced, but not alfaprostol-induced luteolysis, was blocked by cotreatment in vivo with the ACE antagonist captopril. These findings support the hypothesis that PGF2α regulates luteolysis through intraluteal activation of the renin-angiotensin/EDN1 systems in CL that have acquired luteolytic competence.**

ACE, *corpus luteum function,* EDN1, EDNRA, EDNRB, *luteolysis, ovary*

## INTRODUCTION

Luteolysis is a streamlined process that involves functional and structural changes that end with the complete demise of corpora lutea (CL) through apoptotic pathways [1, 2]. In the rabbit, which is a reflex ovulator, the luteolytic mechanism comes into play either at the end of gestation or after an infertile mating around Day 14 of pseudopregnancy [3]. In this species, CL regression is driven by prostaglandin (PG) F2α (PGF2α) of uterine origin [4], given that hysterectomy [5], endometritis [6], and indomethacin treatment [7] prolong luteal function.

Besides the key role of PGF2α, several distinct intraluteal pathways have emerged as potential candidate mediators of its luteolytic action [8]. Prominent among these mediators is endothelin–1 (EDN1), a potent vasoconstrictor that is synthesized by the vascular endothelium [9–11], which inhibits progesterone release both in vivo and in vitro in a number of species [12–15]. Recently, using the pseudopregnant rabbit model in the mid-luteal stage, we have shown that the luteolytic action of EDN1 is mediated by complex interactions with the prostanoid cascade via its receptor subtype EDNRA; for reasons that remain unclear, PGF2α does not require EDN1 to elicit its luteolytic effect [16].

An increasing body of evidence indicates that angiotensin II (ANGII), which is the main effector of the renin-angiotensin system (RAS) that regulates systemic blood pressure and electrolyte balance, may act together with EDN1 in the control of CL life-span [17, 18]. In fact, ANGII is released by midcycle bovine CL in vitro and suppresses progesterone release [19]. Moreover, all the components of the RAS have been identified in the ovaries of rats and cows, including renin and angiotensin I converting enzyme (ACE), the enzymes that catalyze ANGI and ANGII production, respectively, as well as the ANGII peptide and its receptor [17, 20–26]. In addition, between PGF2α and both EDN1 and ANGII, several modulated feedback and auto-amplifying mechanisms have been described in the CL of ruminants [27–29].

However, despite the extensive studies on luteal regression, the physiological relevance of these vasoactive peptides in the control of luteal function is still uncertain. Similarly, little is known about which mechanisms protect the growing CL from the functional luteolysis that occurs in the early luteal stage up to Day 9 of pseudopregnancy, when the CL of rabbits shift from refractoriness to complete responsiveness to exogenous PGF2α [30, 31, 32] and EDN1 [16].

Therefore, the main objective of the present study was to elucidate the basal and PGF2α analogue (alfaprostol)-induced temporal expression changes for early luteal (Day-4) and midluteal stage (Day-9) CL of pseudopregnancy, in order to characterize the role of the EDN1/RAS system in the acquisition of luteal sensitivity to PGF2α. In addition, using in vivo and in vitro studies, we further investigated the luteolytic mechanisms of action of EDN1.

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## MATERIALS AND METHODS

### Reagents

Primers for the mRNAs of *EDN1*, *EDNRA*, *EDNRB*, and *ACE*, random hexamer primers, DNAase I, RNAse H-reverse transcriptase (Superscript II), *E. coli* RNase H, DNA molecular mass ladders, TRIzol, Taq DNA polymerase (Platinum), 10× PCR Buffer, 50 mM $MgCl_2$, RNAse-free tubes, RNAse-free water, and deoxy-NTPs were obtained from Invitrogen (S. Giuliano Milanese, Milan, Italy). Primers for *18S* rRNA and the corresponding competimers (QuantumRNA 18S Internal Standards) were acquired from Ambion (Austin, TX). Tritiated hormones and [2,3–3H]L-arginine, with a specific activity of 30–40 Ci/mmol, were purchased from Amersham Biosciences (Amersham Biosciences Ltd., Little Chalfont, Bucks, UK), while progesterone and PGF2α antisera and nonradioactive hormones were from Sigma (St Louis, MO). The NOS Detect Assay Kit was purchased from Alexis Corp. (Läufelfingen, Switzerland). The primary mouse monoclonal anti-EDN1 antibody, which was used for immunohistochemistry, was supplied by ABR Affinity Bioreagents (Golden, CO). The biotinylated secondary antibody, goat anti-mouse IgG, was purchased from Vector Laboratories (Burlingame, CA). The avidin-biotin complex (ABC, Vector Elite Kit) and the chromogen 3,3′-diaminobenzidine tetrachloride (DAB) were also from Vector Laboratories. The kit for protein assays was purchased from Bio-Rad Laboratories (Hercules, CA).

Incubation wells were obtained from Becton Dickinson (Clifton, NJ), medium 199 and Earles Balanced Salt Solution (EBSS) were from GIBCO (Grand Island, NY). Phospholipase A2 ($PLA_2$, 4-bromophenacyl bromide), PLC (compound 4880), PKC (staurosporine), ACE (captopril) inhibitors, Hepes, and BSA were purchased from Sigma, whereas all other pure grade chemical and reagents were obtained locally. EDN1 was purchased from Calbiochem (San Diego, CA).

The following hormonal preparations were administered via i.m. injection: GnRH analogue (Receptal; Hoechst-Roussel Vet, Milan, Italy), eCG (Folligon; Intervet, Milan, Italy), and the PGF2α analogue alfaprostol (Gabbrostim; Centralvet, Milan, Italy).

### Animals, Hormonal Regimen, and Tissue Collection

The protocols involving the use of the animals in these experiments were approved by the Bioethics Committee of the University of Perugia. Unmated New Zealand White rabbits of 5 mo of age and weighing 3.5–3.8 kg were caged individually in quarters at the University of Perugia Central Animal Facility and maintained under controlled conditions of 14L:10D and 18°C. The animals were provided with commercial rabbit chow and tap water ad libitum. All rabbits were treated with 20 IU eCG, followed 3 days later by i.m. injection of 0.8 μg GnRH (Day 0) to induce pseudopregnancy [33]. The rabbits were maintained in accordance with the standards of animal care.

On Day 4 or Day 9 of pseudopregnancy, 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 analogue administration. Reproductive tracts, which were promptly removed from each animal, were thoroughly washed with saline. For the analyses of luteal gene expression by RT-PCR, the CL were excised from ovaries and after careful dissection of nonluteal tissue using fine forceps under stereoscopic magnification, the specimens were rinsed with RNAse-free PBS and immediately frozen at −80°C. For the in vitro study, CL were harvested from three does that were killed on Days 4 and 9 of pseudopregnancy, as previously reported [31].

For the purpose of the present study, functional luteolysis was defined as a 50% drop in plasma progesterone relative to the pretreatment values, while complete luteolysis was judged to be the failure of CL to secrete progesterone, such that the levels of progesterone in the blood fell below 1.0 ng/ml, which is the concentration detected in estrus rabbits [34].

### Immunohistochemistry of EDN1

For the immunohistochemical detection of EDN1, two additional animals for each time-point were killed just prior (time 0) to alfaprostol injection and 1.5 h and 3 h after alfaprostol injection administered on either Day 4 or Day 9 of pseudopregnancy. The ovaries were immediately excised, fixed by immersion in 4% (v/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. Serial 7-μm-thick sections mounted on poly-L-lysine-coated glass slides were dewaxed in xylene and hydrated through a graded ethanol series. The sections were microwave irradiated for 5 min at 750 W in 10 mM citric acid buffer (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 pseudoperoxidase activity, then rinsed with PBS and incubated for 30 min with normal goat serum, to minimize the nonspecific binding of reagent in the subsequent steps. To reduce variability in staining, within each luteal stage, all the tissue sections were incubated together. The monoclonal mouse anti-EDN1 antibody was raised against a peptide that maps within an internal region of human EDN1, which shows 100% amino acid sequence homology with the same region in the rabbit EDN1. The primary antiserum was diluted 1:100 in PBS and added to the 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, the 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. The slides were then washed in PBS, counterstained with Gill's hematoxylin, and dehydrated before mounting in Canada balsam natural. Positive reactions were recognized as reddish brown precipitates. Sections for which the primary antibody was omitted or substituted by preimmune goat γ-globulin were used as negative controls for nonspecific staining.

### RNA Extraction and Reverse-Transcription Multiplex RT-PCR Amplification

For each rabbit, total RNA was extracted from a pool of eight CL (approximately 90 mg of tissue) and reverse-transcribed into cDNA, while multiplex PCR amplification was carried out with *EDN1*, *EDNRA*, *EDNRB*, and *18S* sense and antisense primers, as reported elsewhere [16]. The primer sequences for *ACE* (168-bp product) were: forward, 5′-CCTCTACCT-GAACCTGC-3′ and reverse, 5′-TGGGCTTGTCTGGAAAGG-3′. Preliminary experiments were carried out to establish the optimal ratio of *18S* primers to their competimers, i.e., primers that were modified only at their 3′-ends to block extension by DNA polymerase, to be used in the reaction mix. The ratios were 2:8 for *EDN1* and *ACE* and 3:7 for *EDNRA* and *EDNRB* RT-PCRs, respectively. The *18S* rRNA is an ideal internal control for quantitative RNA analysis because its expression remains invariant across tissues and treatments, whereas its relatively high abundance makes it difficult to use in RT-PCR experiments, in which target mRNA genes are far less abundant. However, by mixing the appropriate amounts of *18S* primers with their competimers, the PCR amplification efficiency of *18S* cDNA can be reduced to a level that is roughly similar to that of the gene under study. Between 30 and 40 cycles, both the target and *18S* products were in a linear exponential phase of amplification (data not shown). All the PCR reactions consisted of an initial denaturation step at 94°C for 75 sec, followed by amplification for 35 cycles of 94°C for 15 sec, 60°C for 30 sec, and 72°C for 45 sec, with a final extension step of 72°C for 10 min. Within each experiment and for each gene analyzed, 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 were analyzed by electrophoresis on 2% agarose gels using ethidium bromide staining for visualization. The analysis of amplification products was carried out as reported elsewhere [16].

### In Vivo Induction of Luteolysis

EDN1 was dissolved in saline at a concentration of 10 μg/ml and administered i.v. via a catheter inserted in the central ear vein, as previously reported [16]. The ACE inhibitor captopril was dissolved in saline at a concentration of 5 mg/ml and injected s.c. (1 mg/kg) 1 h before EDN1 or PGF2α analogue administration and again 8 h later. Both the dosage and treatment protocols for captopril were based on the recently published experimental data for cows [18].

On Day 9 of pseudopregnancy, rabbits (n = 5 per group) were randomly assigned to receive one of the following treatments: saline (control), EDN1 (10 μg), EDN1 plus ACE inhibitor, alfaprostol (100 μg), and alfaprostol plus ACE inhibitor.

From each rabbit, blood samples were collected by venopuncture of the marginal ear vein just before (basal) and 2, 4, 8, 24, and 48 h after treatment. The samples, which were collected in EDTA-containing tubes, were immediately centrifuged at 3000 × *g* for 15 min, and the plasma was stored frozen until assayed for progesterone concentration, to assess CL functional status.

### In Vitro Incubations

For the in vitro study, Day-4 or Day-9 CL were randomly distributed (one CL/well) into incubation wells in 1 ml of culture medium 199 with EBSS that contained 2.2 mg/ml sodium bicarbonate, 2.3 mg Hepes, and 3% BSA, referred to here as M199. Before treatment, CL were placed inside each well using fine forceps. Each incubation set of wells was divided into five experimental groups

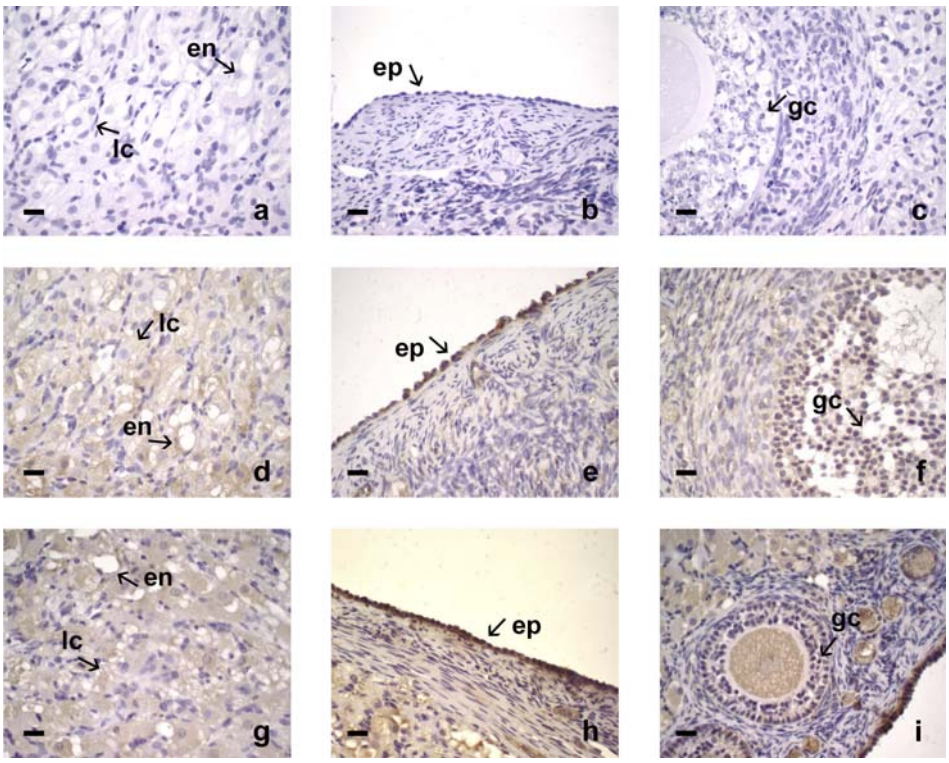


FIG. 1. Cellular immunolocalization of EDN1 in rabbit ovaries collected on Day 4 of pseudopregnancy before (**d**–**f**) and 3 h (**g**–**i**) after PGF2α analogue (alfaprostol) injection, showing positive staining in luteal cells (lc), endothelial cells (en), ovarian epithelium (ep), and granulosa cells of follicles (gc). Control sections (**a**–**c**) incubated in the absence of primary antibody show no reactivities in the lc, en, ep, and gc. Bar = 20 μm.

of five wells as follows: (I) medium alone as control; (II) EDN1 (100 μM); (III) EDN1 plus $PLA_2$ inhibitor (100 nM); (IV) EDN1 plus PLC inhibitor (100 nM); and (V) EDN1 plus PKC inhibitor (100 nM). The culture plates were incubated at 37°C in air with 5% $CO_2$ as reported elsewhere [16]. The medium in each well was collected after 4 h of incubation and stored immediately at −20°C for later determinations of progesterone and PGF2α. Each CL was weighed and stored immediately at −20°C for later determinations of NOS activity and protein concentration. Preliminary evidence, together with the previous results [16], guided our choices of incubation conditions and the minimum effective dosages of the substances used in the present in vivo study (data not shown).

### *Progesterone and PGF2α Assays*

Progesterone and PGF2α concentrations were determined by RIA with specific antibodies, according to the procedures reported elsewhere [32]. The assay sensitivity was 0.08 ng/ml for progesterone and 19 pg/ml for PGF2α. The intraassay and interassay coefficients of variation were 5.3% and 10.2% for progesterone and 8% and 12% for PGF2α, respectively.

### *NO Synthase Activity Determination*

In rabbit CL, NOS activity was determined by monitoring the conversion of [$^3$H]L-arginine into [$^3$H]L-citrulline using a commercial NOS assay kit according to the procedure described previously [32].

### *Statistical Analysis*

At each stage of pseudopregnancy, the ratios of each PCR product for the target genes (*EDN1*, *EDNRA*, *EDNRB*, and *ACE*) normalized against the coamplified *18S* product were analyzed by two-way ANOVA (time after alfaprostol treatment and gel being the two sources of variability) and the Newman-Keuls multi-comparison post-test. Data relative to overall in vivo treatment effects on plasma progesterone during the 48-h time-course of EDN1- or PGF2α analogue-induced luteolysis were analyzed by ANOVA for repeated measurements according to a model that included treatment group, doe within group, time period, and interaction between group and time period; doe within treatment was used as the error term. Progesterone, PGF2α, and enzyme activity from the in vitro data were evaluated by ANOVA. All statistical analyses were performed using Prism (GraphPad Software Inc., San Diego, CA). Comparisons among all the data for days and time-points were evaluated using the Student *t*-test. A value of $P < 0.01$ was considered significant.

## RESULTS

### EDN1 *Immunolocalization in Rabbit CL*

Using a monoclonal antibody, positive staining for EDN1 was localized in the endothelial cells of the luteal blood vessels of ovaries collected before treatment on either Day 4 (Fig. 1d) or Day 9 of pseudopregnancy (Fig. 2d). In the same unstimulated ovaries, cytoplasmic positive signals were also identified in the luteal (Figs. 1 and 2d) and granulosa cells of the follicles (Figs. 1 and 2f), as well as in the ovarian epithelial cells (Figs. 1 and 2e). In each positive cell type, staining was evident 3 h after PGF2α analogue injection independently of luteal stage (Figs. 1 and 2, g–i) and thereafter (data not shown). Staining was completely abolished when the primary antibody was substituted with nonimmune serum (Figs. 1 and 2, a–c).

### *Luteal Gene Expression*

The corresponding amplification products obtained using primers designed for each gene matched the expected sizes (Figs. 3–6, panels B and C).

EDN1 *mRNA*. Prior to treatment, the relative abundances of *EDN1* mRNA did not differ between Day-4 and Day-9 CL (Fig. 3A). At Day 4 of pseudopregnancy, the luteal *EDN1* mRNA levels rose two-fold ($P \leq 0.01$) 1.5 h after alfaprostol treatment, and remained at approximately the same high levels ($P \leq 0.01$) for the following 24 h (Fig. 3A). At Day 9 of pseudopregnancy, the numbers of *EDN1* mRNA luteal transcript were increased ($P \leq 0.01$) 1.5 h and 3 h after PGF2α analogue challenge (Fig. 3A). Thereafter, the numbers of *EDN1* transcript dropped ($P \leq 0.01$) to the pretreatment values (Fig. 3A).

EDNRA *and* EDNRB *mRNAs*. Before treatment, both the *EDNRA* (Fig. 4A) and *EDNRB* (Fig. 5A) mRNA transcripts were poorly expressed in Day-4 CL compared to Day-9 CL ($P \leq 0.01$). Upon alfaprostol administration at Day 4 of pseudopregnancy, both the *EDNRA* and *EDNRB* mRNA levels

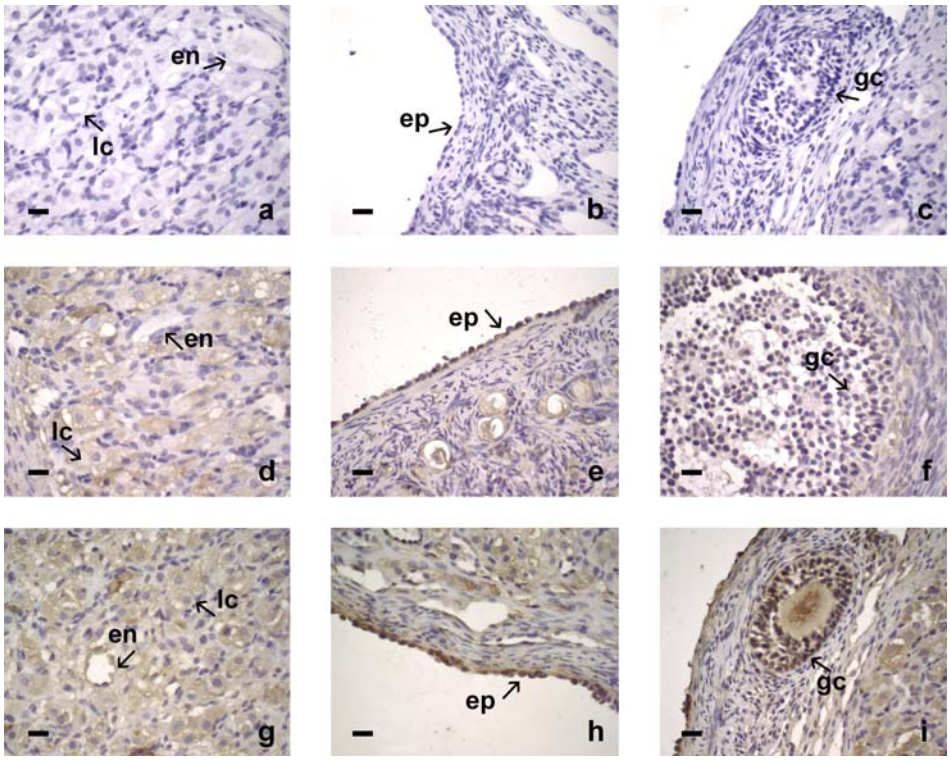


FIG. 2. Cellular immunolocalization of EDN1 in rabbit ovaries collected on Day 9 of pseudopregnancy before (**d–f**) and 3 h (**g–i**) after PGF2α analogue (alfaprostol) injection, showing positive staining in luteal cells (lc), endothelial cells (en), ovarian epithelium (ep), and granulosa cells of follicles (gc). Control sections (**a–c**) incubated in the absence of primary antibody show no reactivities in the lc, en, ep, and gc. Bar = 20 μm.

were increased ($P \leq 0.01$) two- to three-fold, and remained high ($P \leq 0.01$) for the following 24 h (Figs. 4 and 5A). Conversely, at Day 9 of pseudopregnancy, the luteal *EDNRA* and *EDNRB* genes did not show any clear patterns, remaining at approximately the same levels for up to 24 h after PGF2α analogue challenge (Figs. 4 and 5A).

ACE *mRNA*. Before treatment, the steady-state level of *ACE* mRNA was lower ($P \leq 0.01$) at Day 4 than at Day 9 of pseudopregnancy (Fig. 6A). At Day 4, the *ACE* mRNA levels rose almost two-fold ($P \leq 0.01$) within the first 1.5 h after alfaprostol administration and remained at approximately the same relatively high levels ($P \leq 0.01$) for the following 24 h (Fig. 6A). Conversely, at Day 9 of pseudopregnancy, the relative abundance of *ACE* mRNA increased ($P \leq 0.01$) 1.5 h after alfaprostol challenge, but then declined to basal values during the course of luteal regression (Fig. 6A).

### *In Vitro Culturing of CL*

For Day-9 CL cultured in vitro, EDN1 decreased ($P \leq 0.01$) progesterone release (Fig. 7A) but increased ($P \leq 0.01$) PGF2α secretion (Fig. 7B) and NOS activity (Fig. 7C). Coincubation of EDN1 with the $PLA_2$-inhibitor did not modify the EDN1-

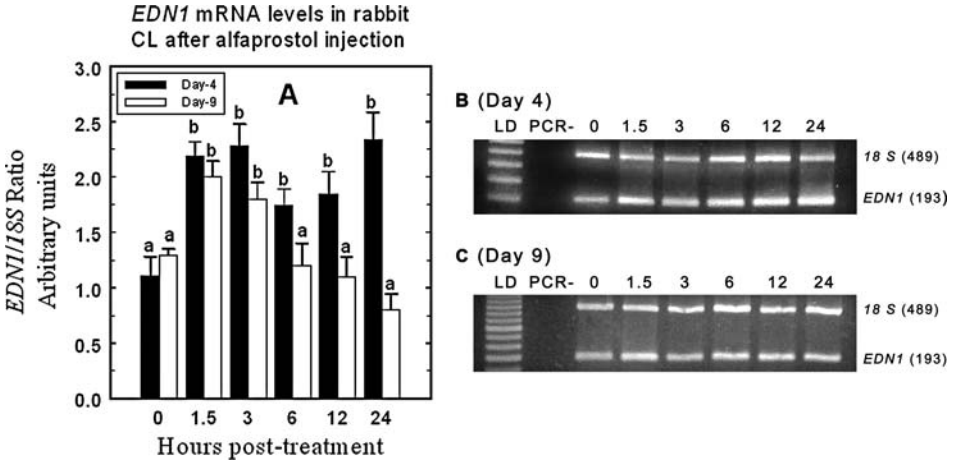


FIG. 3. Gene expression patterns of *EDN1* mRNAs in CL of rabbits collected 0, 1.5, 3, 6, 12, and 24 h after PGF2α analogue (alfaprostol) injection on Day 4 or Day 9 of pseudopregnancy. **A**) Summarized data (means ± SEM) derived from densitometric analyses of *EDN1* in CL, reported in arbitrary units relative to *18S* expression. For each day of pseudopregnancy and for each time-point, the values shown represent the combined results for three different rabbits. Different letters above the bars indicate significantly different values ($P < 0.01$) among treatment days and time-points. **B**, **C**) Representative photographs (Day 4, **B**; Day 9, **C**) of typical 2% agarose, ethidium bromide-stained gels, showing the presence of the expected products yielded after RT-PCR using primers for the target *EDN1* and *18S*. Lane LD shows the kilobase DNA marker, lane PCR represents a negative control of RNA that was not reverse-transcribed but was subjected to PCR amplification, while the other lanes represent samples taken at the corresponding hours after alfaprostol injection.

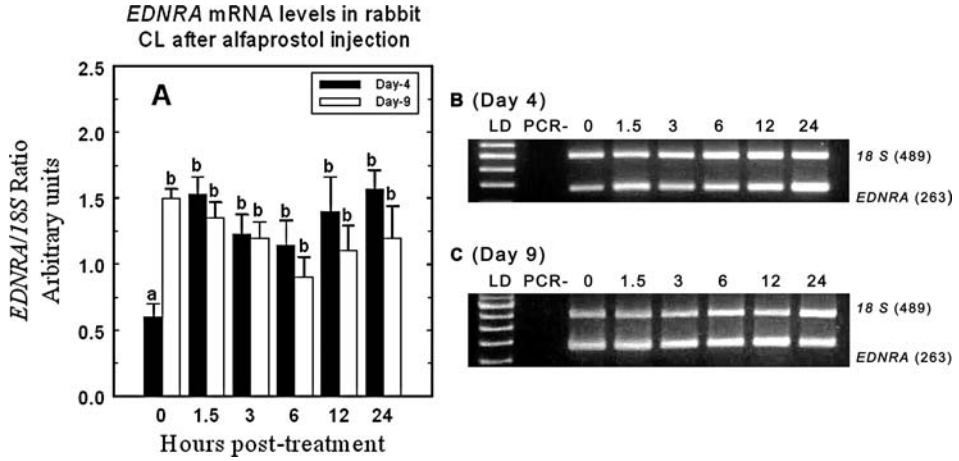


FIG. 4. Gene expression patterns of *EDNRA* mRNAs in CL of rabbits collected 0, 1.5, 3, 6, 12, and 24 h after PGF2α analogue (alfaprostol) injection on Day 4 or Day 9 of pseudopregnancy. **A**) Summarized data (means ± SEM) derived from densitometric analyses of *EDNRA* in CL, reported in arbitrary units relative to *18S* expression. For each day of pseudopregnancy and for each time-point, the values shown represent the combined results for three different rabbits. Different letters above the bars indicate significantly different values ($P < 0.01$) among days and time-points. **B**, **C**) Representative photographs (Day 4, **B**; Day 9, **C**) of typical 2% agarose, ethidium bromide-stained gels, showing the presence of the expected products yielded after RT-PCR using primers for the target *EDNRA* and *18S*. Lane LD shows the kilobase DNA marker, lane PCR represents a negative control of RNA that was not reverse-transcribed but was subjected to PCR amplification, while the other lanes represent samples taken at the corresponding hours after alfaprostol injection.

dependent effects either on hormone or NOS activity (Fig. 7). Conversely, the addition of either PLC or PKC inhibitors blocked the luteolytic action of EDN1 on both progesterone and PGF2α release, as well as on NOS activity, all of which remained at control values (Fig. 7). In Day-4 CL, EDN1 did not cause any changes among the experimental groups in terms of hormone release and enzyme activity (data not shown).

### *In Vivo Induction of Luteolysis*

The plasma P4 concentrations, which are used as a marker of luteal functional activity, decreased similarly in rabbits 8 h after EDN1 or alfaprostol injection on Day 9 of pseudopregnancy, and complete functional luteolysis was achieved 24 h later when the plasma P4 levels dropped to 0.3 ng/ml in both groups (Fig. 8). EDN1-induced functional luteolysis was blocked by pretreatment with the ACE inhibitor captopril (Fig. 8). Conversely, alfaprostol-induced luteolysis was not affected by cotreatment with captopril (Fig. 8).

## DISCUSSION

The present study provides evidence that exogenous PGF2α differentially modulates the expression of *EDN1*, its receptors, and *ACE* in both the early luteal- and midluteal-phase CL of pseudopregnant rabbits. However, only in the latter, which have acquired luteolytic competence, does a positive and reciprocal feed-back mechanism exist between PGF2α and EDN1, likely mediated by ACE, come into play to induce functional and structural luteolysis.

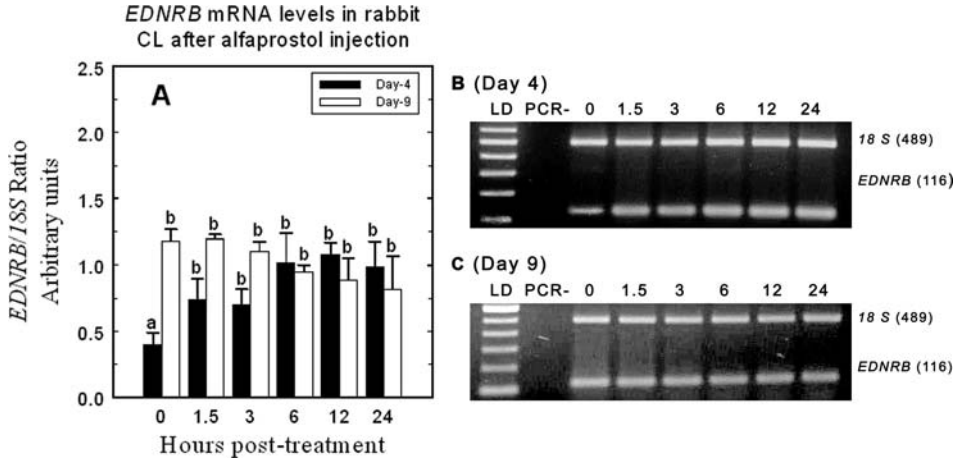


FIG. 5. Gene expression patterns of *EDNRB* mRNAs in the CL of rabbits collected 0, 1.5, 3, 6, 12, and 24 h after PGF2α analogue (alfaprostol) injection on Day 4 or Day 9 of pseudopregnancy. **A**) Summarized data (means ± SEM) derived from densitometric analyses of *EDNRB* in CL, reported in arbitrary units relative to *18S* expression. For each day of pseudopregnancy and for each time-point, the values shown represent the combined results for three different rabbits. Different letters above the bars indicate significantly different values ($P < 0.01$) among days and time-points. **B**, **C**) Representative photographs (Day 4, **B**; Day 9, **C**) of typical 2% agarose, ethidium bromide-stained gels, showing the presence of the expected products yielded after RT-PCR using primers for the target *EDNRB* and *18S*. Lane LD shows the kilobase DNA marker, lane PCR represents a negative control of RNA that was not reverse-transcribed but was subjected to PCR amplification, while the other lanes represent samples taken at the corresponding hours after alfaprostol injection.

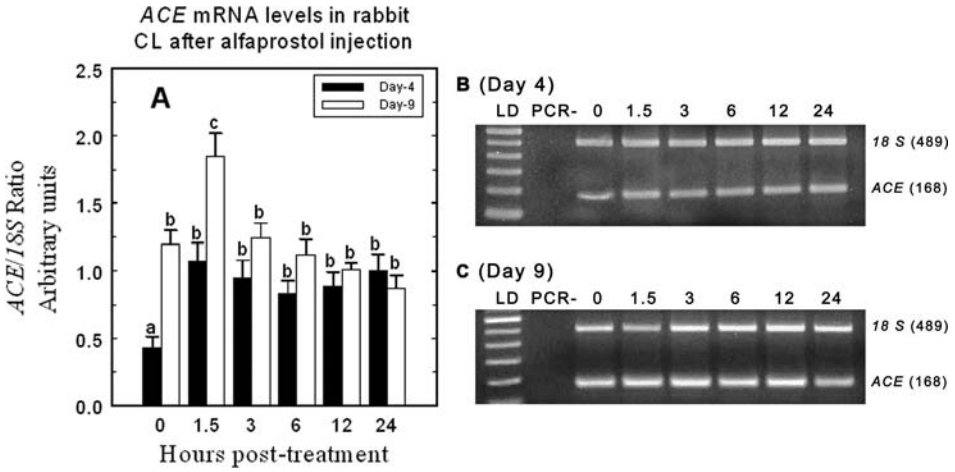


FIG. 6. Gene expression patterns of *ACE* mRNAs in the CL of rabbits collected 0, 1.5, 3, 6, 12, and 24 h after PGF2α analogue (alfaprostol) injection on Day 4 or Day 9 of pseudopregnancy. **A**) Summarized data (means ± SEM) derived from densitometric analyses of *ACE* in CL, reported in arbitrary units relative to *18S* expression. For each day of pseudopregnancy and for each time-point, he values shown represent the combined results for three different rabbits. Different letters above the bars indicate significantly different values ($P \leq 0.01$) among days and time-points. **B**, **C**) Representative photographs of typical 2% agarose, ethidium-bromide stained gels, showing the presence of the expected products yielded after RT-PCR using primers for the target *ACE* (168 bp) and *18S* (489 bp). Lane LD shows the kilobase DNA marker, lane PCR represents a negative control of RNA that was not reverse-transcribed but was subjected to PCR amplification, while the other lanes represent samples taken at the corresponding hours after alfaprostol injection.

The cell-type distribution of EDN1 within the rabbit ovary was precisely identified by the IHC technique. Independently of the luteal stage examined, either before or after PGF2α treatment, EDN1 staining was clearly evidenced in the vascular endothelial and luteal cells of the CL. In contrast, Schams et al. [18], using the IHC technique on bovine midcycle CL, revealed no or only very weak staining for EDN1 in both endothelial and steroidogenic cells, before and up to 4 h after PGF2α injection. This discrepancy is likely due to interspecies differences and/or to methodological causes rather than to the specificity of the primary antibody used. In fact, the mature forms of EDN1 are highly conserved among species, and the rabbit *EDN1* nucleotide sequence is identical to that of human, porcine, rat, bovine, and canine origin [35]. In the present study, positive immunoreactivity for EDN1 was evidenced in the cytoplasm of follicular granulosa cells, as previously found for other species [36–40], and in the ovarian surface epithelial cells, which are the sites of an inflammatory-like response during ovulation [41, 42].

Before treatment, the relative abundance of *EDN1* mRNA was comparable between young and mature CL, whereas those of the *EDNRA*, *EDNRB*, and *ACE* mRNAs were much lower in Day 4 than in Day 9 CL. These results confirm that all the *EDN1* system members and *ACE* are constitutively expressed, albeit at different levels, in rabbit CL, as reported in other species [43]. These differences may reflect developmental changes in the ratio between steroidogenic and endothelial cell compartments over the total cell population of rabbit CL [44] or in their gene expression levels. Subtle differences in the intraovarian hormonal milieu during the luteal growing phase [11] could also be responsible for the discrepancy with the previous report [16] in terms of the relative abundances of *EDN1* and *EDNRB* transcripts in Day-4 CL.

In the present study, exogenous PGF2α analogue challenge clearly up-regulated the transcription of both the *EDN1* and *ACE* genes, independently of luteal stage and well in advance of progesterone decline [45]. Interestingly, whereas the expression levels of these mRNAs remained high and quite

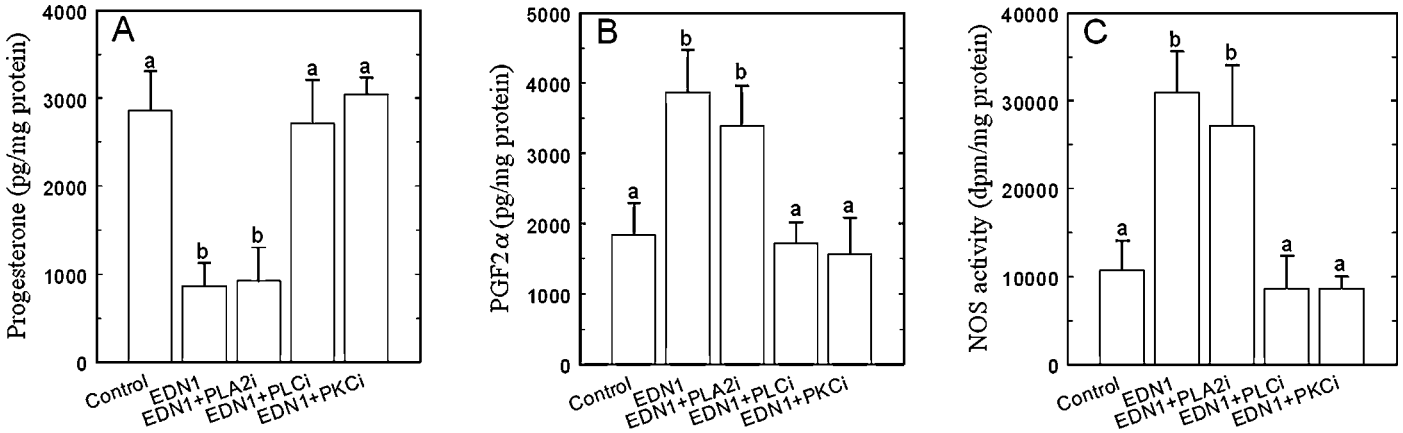


FIG. 7. In vitro effects of medium alone, endothelin 1 (EDN1), EDN1 plus PLA2 inhibitor, EDN1 plus PLC inhibitor, and EDN1 plus PKC inhibitor on both progesterone (**A**) and PGF2α (**B**) release, and total NOS activity (**C**) by rabbit CL harvested on Day 9 of pseudopregnancy. The results shown are the means ± SD of five replicates. Different letters above the bars indicate significantly different values ($P \leq 0.01$).

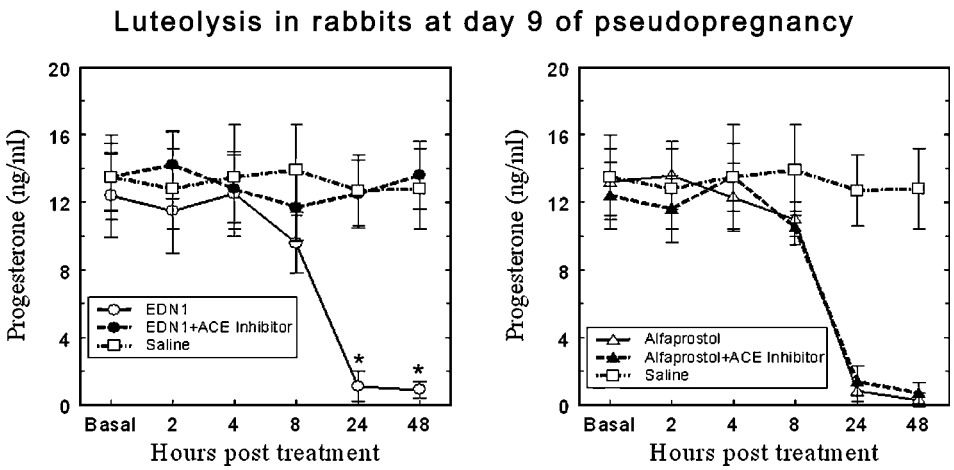


FIG. 8. In vivo effects of EDN1 (10 μg) and EDN1 plus ACE inhibitor (captopril, 1 mg/kg s.c. given twice) (left panel), and PGF2α analogue (alfaprostol, 100 μg) and PGF2α plus captopril (right panel) on progesterone plasma levels at Day 9 of pseudopregnancy. Values are mean ± SEM for 5 animals/group. *, $P \leq 0.01$ compared to the control (saline).

stable in young CL for the 24 h following PGF2α treatment, in mature CL they were up-regulated only for the first 1.5–3 h. PGF2α up-regulated both the *EDNRA* and *EDNRB* genes in the early-stage CL, whereas no modulation was evident in the midluteal-stage CL, probably because they were already being expressed at their maximal levels. On the other hand, the basal *EDNRA* and *EDNRB* mRNA levels in the midluteal CL were similar to those induced by PGF2α in the early luteal CL. The *EDN1*, *EDNRA*, *EDNRB*, and *ACE* expression differences suggest that the luteal cell components of growing and mature CL react differently to the same dosage of exogenous PGF2α, which supports the idea of a functional role for the RAS/EDN1 system in the luteolytic cascade evoked by PGF2α. However, it remains to be established whether the stimulatory action of PGF2α on the rabbit CL *EDN1* gene is exerted directly or indirectly, given that in bovine carotid artery endothelial cells, ANGII immediately and dose-dependently up-regulates the expression of preproendothelin 1 mRNA [46]. However, the insensitivity of CL to the luteolytic action of PGF2α in the early luteal stage cannot be ascribed to a faulty response of the local EDN1/RAS system activation, but rather to some other mechanism, including the lack of endogenous PGF2α production which modulates CL function, as previously indicated for other species [47, 48] and for rabbits [49].

PGF2α-induced *EDN1* gene expression increases have also been observed in midluteal-phase CL of cycling ewes [15] and cows [10, 11, 18, 50, 51], but not in early luteal-phase bovine CL [11, 52]. In cattle, PGF2α injection rapidly increases EDN1 release within the regressing CL, as well as into the ovarian venous blood [53]. Furthermore, the regulatory mechanisms that control the expression of *ACE* have been demonstrated in bovine CL during the estrus cycle and after the induction of luteal regression, when luteal *ACE* mRNA expression increases continuously, peaking 12 h and 24 h after PGF2α injection on Days 8 and 12 of the estrus cycle [17, 18, 54]. Interestingly, in cows, the overall response of CL EDN1 receptors to the luteolytic challenge dose of PGF2α showed the opposite pattern, since on Day 4 it failed to up-regulate *EDNRA* [11] but was effective at up-regulating both subtypes for up to 48 h when administered on Days 8–10 of the cycle. Thus, in contrast to what has been found in cows [11, 52], the EDN1 system in the CL of rabbits during the early luteal phase are not completely refractory to PGF2α, even if it is not able to decrease progesterone production.

Our in vitro results show that EDN1, after engagement of its cognate binding site, likely EDNRA [16], activates the G protein-dependent PLC/PKC pathway, which inhibits progesterone release and enhances PGF2α synthesis and NOS activity in Day-9 CL. These data are in agreement with previous reports on EDN1 intracellular signaling systems in other tissues [55, 56], as well as in ovine, bovine, and human luteal cells [15, 52, 57, 58]. In contrast, EDN1 did not affect the basal production levels of progesterone and PGF2α or NOS activity in Day-4 CL, in agreement with our previous results obtained in vivo [16]. These findings can be justified by the relatively low levels of receptors for EDN1, as compared to those present in Day-9 CL; alternatively, they can be explained by changes in the EDN1 intracellular signaling pathways, rather than to reduced or impaired synthesis of EDN1, since PGF2α up-regulates its expression. In bovine CL, Choudhary et al. [59] found that the luteolytic efficacy of EDN1 was the same on Days 4 and 10, suggesting that this vasoactive agent is a tonic inhibitor of progesterone production rather than a mediator of PGF2α action. These contrasting findings suggest that the mechanism of acquisition of luteolytic competence is different in rabbit and bovine CL.

In the present study, we have shown that EDN1-induced luteolysis at Day 9 of pseudopregnancy is inhibited by antagonizing ACE with captopril. Previously, we found that the luteolytic action of EDN1 was also blocked by pretreatment with indomethacin, a cyclooxygenase inhibitor [16]. Taken together, these findings further support the putative role of the RAS/EDN1 system in the luteolytic process, which is probably also mediated by the prostanoid pathway in the rabbit, similar to what has been described in cows [18, 23, 60]. However, our present data show that PGF2α-induced luteolysis is not affected by the blockade of ACE, which confirms that, at least in the pseudopregnant rabbit model, the cascade mechanism triggered by the prostaglandin analogue alfaprostol does not require the RAS system for the stimulation of luteolysis, in accordance with previous results obtained for the cow [18].

The findings of the present study, together with the results of previous studies conducted by us [16] and others [48, 61, 62], suggest that in midluteal-phase CL of pseudopregnant rabbits, positive reciprocal feedback mechanisms exist between RAS/EDN1 and PGF2α, which are capable of inducing functional and structural luteolysis. With a short time-lag, exogenous PGF2α stimulates endothelial and luteal cells to express increasing levels of EDN1 transcripts. EDN1, via autocrine and paracrine mechanisms, engages luteal EDNRA receptors and stimulates, through ACE mediation, the intraluteal synthesis of new PGF2α, which in turn binds to its luteal FP receptors and up-regulates EDN1 expression in both endothelial and luteal cells in both paracrine and autocrine

fashions. Although the present data add new pieces to the biological puzzle of luteolysis in the rabbit model, further studies are needed to improve our understanding of the system of fine tuning that controls the CL life span in this species and the differential, age-dependent resistance to the luteolytic action of exogenous PGF2α.

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## REFERENCES

1. McCracken JA, Custer EE, Lamsa JC. Luteolysis: a neuroendocrine-mediated event. Physiol Rev 2000; 70:263–323.
2. 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.
3. Carlson JC, Gole JWD. CL Regression in the pseudopregnant rabbit and the effects of treatment with prostaglandin F-2α and arachidonic acid. J Reprod Fert 1978; 53:381–387.
4. Lytton FDC, Poyser NL. Concentrations of PGF-2α and PGE-2 in the uterine venous blood of rabbits during pseudopregnancy and pregnancy. J Reprod Fertil 1982; 64:421–429.
5. Scott RS, Rennie PIC. Factors controlling the life span of the corpora lutea in the pseudopregnant rabbit. J Reprod Fer 1970; 23:415–422.
6. Boiti C, Canali C, Brecchia G, Zanon F, Facchin E. Effects of induced endometritis on the life-span of corpora lutea in pseudopregnant rabbits and incidence of spontaneous uterine infections related to fertility of breeding does. Theriogenology 1999; 52:1123–1132.
7. O'Grady JP, Caldwell Bv, Auletta FJ, Speroff L. The effects of an inhibitor of prostaglandin synthesis (indomethacin) on ovulation, pregnancy and pseudopregnancy in the rabbit. Prostaglandins 1972; 1:97–106.
8. Webb R, Woad KJ, Armstrong DG. Corpus luteum (CL) function: local control mechanism. Dom Anim Endocrinol 2002; 23:277–285.
9. Usuki S, Suzuki N, Matsumoto H, Yanagisawa M, Masaki T. Endothelin-1 in luteal tissue. Mol Cell Endocrinol 1991; 80:147–151.
10. Meidan R, Milvae RA, Weiss S, Levy N, Friedman A. Intraovarian regulation of luteolysis. J Reprod Fert Suppl 1999; 54:217–228.
11. Meidan R, Levy N. Endothelin-1 receptors and biosynthesis in the corpus luteum: molecular and physiological implications. Dom Anim Endocrinol 2002; 23:287–298.
12. Kamada S, Blackmore PF, Kubota T, Ochninger S, Assada Y, Gordon K, Hodgen GD, Aso T. The role of endothelin-1 in regulating human granulosa cell proliferation and steroidogenesis in vitro. Clin Endocr Metab 1995; 80:3708–3714.
13. Miyamoto A, Kobayashi S, Arata S, Ohtani, Fujui Y, Schams D. Prostaglandin F2α promotes the inhibitory effects of endothelin-1 on the bovine luteal function in vitro. J Endocrinol 1997; 152:R7–R11.
14. Mamluk R, Levy N, Rueda B, Davis JS, Meidan R. Characterization and regulation of type A endothelin receptor gene expression in bovine luteal cell types. Endocrinology 1999; 140:2110–2116.
15. Hinckley ST, Milvae RA. Endothelin-1 mediates prostaglandin F2α–induced luteal regression in the ewe. Biol Reprod 2001; 64:1619–2001.
16. 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.
17. Schams D. The expression of angiotensin and endothelin system members in bovine corpus luteum during estrous cycle and pregnancy. Endocrine 2002; 19:305–312.
18. Schams D, Berisha B, Neuvians T, Amselgruber W, Kraetzl WD. Real-time changes of the local vasoactive peptide systems (angiotensin, endothelin) in the bovine corpus luteum after induced luteal regression. Mol Reprod Develop 2003; 65:57–66.
19. Hayashi K, Miyamoto A. Angiotensin II interacts with prostaglandin F2alpha and endothelin 1 as a local luteolytic factor in the bovine corpus luteum in vitro. Biol Reprod 1999; 60:1104–1109.
20. Husain A, Bumpus FM, DeSilva P, Speth RC. Localization of angiotensin II receptors in ovarian follicles and the identification of angiotensin II in rat ovaries. Proc Natl Acad Sci U S A 1987; 84:2489–2493.
21. Speth RC, Husain A. Distribution of angiotensin-converting enzyme and angiotensin II-receptor binding sites in the rat ovary. Biol Reprod 1988; 38:695–702.
22. Schultze D, Brunswig B, Mukhopadhyay AK. Renin and prorenin-like activities in bovine ovarian follicles. Endocrinology 1989; 124:1389–1398.
23. Usuki S, Saitoh T, Saitoh M, Tanaka J, Kawakura Y, Usuki Y, Shinjo M, Kim SJ. Endothelin-renin-angiotensin-atrial natriuretic peptide system in ovaries: an intraovarian ERAANP system. J Cardiovasc Pharmacol 1993; 22:207–210
24. Kobayashi S, Berisha B, Amselgruber WM, Schams D, Miyamoto A. Production and localisation of angiotensin II in the bovine early corpus luteum: a possible interaction with luteal angiogenic factors and prostaglandin F2 alpha. J Endocrinol 2001; 170:369–380.
25. Schauser KH, Nielsen AH, Winther H, Dantzer V, Poulsen K. Localization of the renin-angiotensin system in the bovine ovary: cyclic variation of the angiotensin II receptor expression. Biol Reprod 2001; 65: 1672–1680.
26. Kobayashi S, Acosta TJ, Ozawa T, Hayashi K, Berisha B, Othani M, Schams D, Miyamoto A. Intraluteal release of angiotensin II and progesterone in vivo during corpora lutea development in the cow: effect of vasoactive peptides. Biol Reprod 2002; 66:174–179.
27. Diaz FJ, Anderson LE, WU YL, Rabot A, Tsai SJ Wiltbank MC. Regulation of progesterone and prostaglandin $F_{2\alpha}$ production in the CL. Mol Cell Endocrinol 2002; 191:65–80.
28. Ohtani M, Takase S, Wijayagunawardane MPB, Tetsuka M, Miyamoto A. Local interaction of prostaglandin F-2 alpha with endothelin-1 and tumor necrosis factor-alpha on the release of progesterone and oxytocin in ovine corpora lutea in vivo: a possible implication for luteolytic cascade. Reproduction 2004; 127:1117–124.
29. Acosta TJ, Miyamoto A. Vascular control of ovarian function: ovulation, corpus luteum formation and regression. Anim Reprod Sci 2004; 82–83: 127–140.
30. Boiti C, Canali C, Zerani M, Gobbetti A. Changes in refractoriness of rabbit corpora lutea to a prostaglandin F2α analogue, alfaprostol, during pseudopregnancy. Prostaglandins 1998; 56:255–264.
31. 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.
32. 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 E-2 and prostaglandin F-2α via adenylate cyclase and phospholipase C. J Endocrinol 2000; 164:179–186.
33. 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; 3:143–148.
34. Browning JY, Keyes PF, Wolf RC. Comparison of serum progesterone, 20α-dihydroprogesterone, and estradiol-17β in pregnant and pseudopregnant rabbits: Evidence for postimplantation recognition of pregnancy. Biol Reprod 1980; 23:1014–1019.
35. Marsden PA, Sultan P, Cybulsky M, Gimbrone MA Jr, Brenner BM, Collins T. Nucletotide sequence of endothelin-1 cDNA from rabbit endothelial cells. Biochim Biophys Acta 1992; 1129:249–250.
36. Kamada S, Kubota T, Taguchi M, Aso T. High levels of immunoreactive endothelin-1 in human follicular fluids. Hum Reprod 1993; 8:674–677.
37. Kubota T, Kamada S, Aso T. Endothelin-1 as a local ovarian regulator in porcine granulosa cells. Horm Res 1994; 41:29–35.
38. Magini A, Granchi S, Orlando C, Vannelli GB, Pellegrini S, Milani S, Grappone C, De Franco R, Susini T, Forti G, Maggi M. Expression of endothelin-1 gene and protein in human granulosa cells. J Clin Endocrinol Metab 1996; 81:1428–1433.
39. Flores JA. Gene expression of endothelin-1 in the porcine ovary: follicular development. Biol Reprod 2000; 63:1377–1382.
40. Acosta TJ, Ozawa T, Kobayashi S, Hayashi K, Ohtani M, Kraetzl WD, Sato K, Schams D, Miyamoto A. Periovulatory changes in the local release of vasoactive peptides, prostaglandin $F_{2\alpha}$, and steroid hormones from bovine mature follicles in vivo. Biol Reprod 2000; 63:1253–1261.
41. Espey LL, Norris C, Saphire D. Effect of time and dose of indomethacin on follicular prostaglandins and ovulation in the rabbit. Endocrinology 1986; 119:746–754.
42. Rae MT, Niven D, Critchley HO, Harlow CR, Hillier SG. Antiinflammatory steroid action in human ovarian surface epithelial cells. J Clin Endocrinol Metab 2004; 89:4528–4544.
43. Berisha B, Schams D, Miyamoto A. The expression of angiotensin and endothelin system members in bovine corpus luteum during estrous cycle and pregnancy. Endocrine 2002; 19:305–312.
44. Dharmarajan AM, Mastroyannis C, Yoshimura Y, Atlas SJ, Wallacj EE, Zirkin BR. Quantitative light microscopic analysis of corpus luteum

growth during pseudopregnancy in the rabbit. Biol Reprod 1988; 38:863–870.
45. Boiti C, Guelfi G, Zerani M, Zampini D, Brecchia G, Gobbetti A. Expression patterns of cytokines, p53, and nitric oxide synthase isoenzymes in corpora lutea of pseudopregnant rabbits during spontaneous luteolysis. Reproduction 2004; 127:229–238.
46. Imai T, Hirata Y, Emori T, Yanagisawa M, Masaki T, Marumo F. induction of endothelin-1 gene by angiotensin and vasopressin in endothelial cells. Hypertension 1992; 19:753–757.
47. Hayashi K, Acosta TJ, Berisha B, Kowayashi S, Ohtani M, Schams D, Miyamoto A. Changes in prostaglandin secretion by the regressing bovine corpus luteum. Prostaglandins Other Lipid Mediat 2003; 70:339–349.
48. 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.
49. Zerani M, Maranesi M, Dall'Aglio C, Brecchia G, Gobbetti A, Boiti C.Age-dependent intraluteal regulation of prostaglandin biosynthesis during PGF2α-induced luteolysis in pseudopregnant rabbits. Reprod Domest Anim 2006; 41:P199,359.
50. Girsh E, Milvae RA, Wang W, Meidan R. Effect of endothelin-1 on bovine luteal cell function: role in prostaglandin F2α–induced antisteroidogenic action. Endocrinology 1996; 137:1306–1312.
51. Wright MF, Savre B, Keith Inskeep EK, Flores JA. Prostaglandin F(2alpha) regulation of the bovine corpus luteum endothelin system during the early and midluteal phase. Biol Reprod 2001; 65:1710–1717.
52. Levy N, Kobayashi S, Roth Z, Wolfenson D, Miyamoto A, Meidan R. Administration of Prostaglandin F2α during the early bovine luteal phase does not alter the expression of ET-1 and its type A receptor: a possible cause for corpus luteum refractoriness. Biol Reprod 2000; 63:377–382.
53. Ohtani M, Kobayashi S, Miyamoto A, Hayashi K, Fukui Y. Real-time relationships between intraluteal and plasma concentrations of endothelin, oxytocin, and progesterone during prostaglandin F2alpha-induced luteolysis in the cow. Biol Reprod 1998; 58:103–108.
54. Kaczmarek MM, Schams D, Ziecik AJ. Role of vascular endothelial growth factor in ovarian physiology – an overview. Reprod Biol 2005; 5: 111–136.
55. Liu Y, Geisbuhler B, Jones AW. Activation of multiple mechanisms including phospholipase S by endothelin-1 in rat aorta. Am J Physiol 1992; 262:C941–C949.
56. Masaki T. Historical review: Endothelin. Trends Pharmacol Sci 2004; 25: 219–224
57. Girsh E, Wang W, Mamluk R, Arditi F, Friedman A, Milvae RA, Meidan R. Regulation of endothelin-1 expression in the bovine corpus luteum: elevation by prostaglandin F2 alpha. Endocrinology 1996; 137:5191–5190.
58. Miceli F, Minici F, Garcia PM, Navarra P, Proto C, Mancuso S, Lanzone A, Apa R. Endothelins enhance prostaglandin (PGE(2) and PGF(2α)) biosynthesis and release by human luteal cells. Evidence of a new paracrine/autocrine regulation of luteal function. J Clin Endocr Metab 2001; 86:811–817.
59. Choudhary E, Sen A, Inskeep EK, Flores JA. Developmental sensitivity of the bovine corpus luteum to prostaglandin F2alpha (PGF2alpha) and endothelin-1 (ET-1): is ET-1 a mediator of the luteolytic actions of PGF2alpha or a tonic inhibitor of progesterone secretion? Biol Reprod 2005; 72:633–642.
60. Shirasuna K, Watanabe S, Oki N, Wijayagunawardane MP, Matsui M, Ohtani M, Miyamoto A. A cooperative action of endothelin-1 with prostaglandin F(2alpha) on luteal function in the cow. Domest Anim Endocrinol 2006; 31:186–196.
61. Shirasuna K, Asaoka H, Acosta TJ, Wijayagunawardane MP, Matsui M, Ohtani M, Miyamoto A. Endothelin-1 within the corpus luteum during spontaneous luteolysis in the cow: local interaction with prostaglandin F2alpha and angiotensin II. J Cardiovasc Pharmacol 2004; 44:252–255.
62. Meidan R, Levy N, Kisliouk T, Podlovny L, Rusiansky M, Klipper E. The yin and yang of corpus luteum-derived endothelial cells: balancing life and death. Domestic Anim Endocrinol 2005; 29:318–328.