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Expression patterns of cytokines, p53 and nitric oxide synthase isoenzymes in corpora lutea of pseudopregnant rabbits during spontaneous luteolysis

Cristiano Boiti, Gabriella Guelfi, Massimo Zerani[1], Danilo Zampini, Gabriele Brecchia and Anna Gobbetti[1]

Dipartimento di Scienze Biopatologiche Veterinarie, Università di Perugia, S. Costanzo 4, 06126 Perugia, Italy and [1]Dipartimento di Biologia Molecolare, Cellulare e Animale, Università di Camerino, Camerino, Italy

Correspondence should be addressed to C Boiti; Email: cristiano.boiti@unipg.it

Abstract

The gene expressions for macrophage chemoattractant protein-1 (MCP-1), interleukin (IL)-1β, IL-2 and p53 were examined by semi-quantitative RT-PCR in corpora lutea (CL) of rabbits during spontaneous luteolysis at days 13, 15, 18 and 22 of pseudopregnancy. In the same luteal tissue, total activity of nitric oxide (NO) synthase (NOS) and genes for both endothelial (eNOS) and inducible (iNOS) isoforms were also analysed. From day 13 to 15, MCP-1 and IL-1β mRNA levels rose ($P \leq 0.01$) almost 2-fold, and the transcript for p53 almost 8-fold, but then all dropped ($P \leq 0.05$) from day 18 onward. IL-2 mRNA abundance was higher ($P \leq 0.01$) on day 13 and then gradually declined. During luteolysis, eNOS mRNA decreased 40% ($P \leq 0.05$) by day 15, but thereafter remained unchanged, while iNOS mRNA was barely detectable and did not show any clear age-related pattern throughout the late luteal stages. Total NOS activity progressively increased ($P \leq 0.01$) from day 13 to 18 of pseudopregnancy and then dropped to the lowest ($P \leq 0.01$) levels on day 22. Luteal progesterone content also declined during CL regression from 411 to 17 pg/mg found on days 13 and 22 respectively, in parallel with the decrease in blood progesterone concentrations. These data further support a physiological role of NO as modulator of luteal demise in rabbits. Locally, luteal cytokines may be involved in the up-regulation of NOS activity, while downstream NO may inhibit steroroidogenesis and induce expression of p53 gene after removal of the protective action of progesterone.

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Introduction

Luteolysis involves both functional and structural changes along a complex and streamlined process that ends with the complete demise of corpora lutea (CL). In rabbits, a reflex ovulator, this mechanism comes into play either at the end of gestation or after an infertile mating to shorten the life span of unnecessary CL and reduce the length of pseudopregnancy (Carlson & Gole 1978). In this species, luteolysis normally begins on approximately day 12–14 of pseudopregnancy and is completed around day 18 when progesterone declines to the basal value (Browning *et al.* 1980).

In rabbits, several factors are now recognised to regulate, via paracrine and/or autocrine mechanisms, the life span of CL from formation to regression (Niswender *et al.* 2000, Webb *et al.* 2002). Among these, convincing evidence suggests that nitric oxide (NO), produced by the action of different NO synthase (NOS) isoenzymes (Schmidt & Walter 1994), may modulate different ovarian functions and luteal regression (Dixit & Parvizi 2001, Tamanini *et al.* 2002). In rabbits, NO inhibits *in vitro* progesterone release by CL at different stages of pseudopregnancy (Gobbetti *et al.* 1999), and NOS activity is up-regulated by prostaglandin F-2α (PGF2α) via the phospholipase C/protein kinase C pathway (Boiti *et al.* 2000). More recently, a number of reports on the regulation of both NOS activity and expression of genes encoding endothelial and inducible NOS isoforms (eNOS and iNOS) during both luteal development (Boiti *et al.* 2002) and PGF2α-induced luteolysis (Boiti *et al.* 2003) in rabbits have been produced. Collectively, these studies provided the first evidence that NO may serve as a regulator of luteal steroidogenesis and act as a potential effector of luteal regression prematurely induced by PGF2α. Moreover, NO deficiency via long-term treatment with L-NAME, a NOS inhibitor, partially blocked the regression mechanism induced by PGF2α and extended luteal function, thus indirectly confirming the role of NO in the luteolytic process (Boiti *et al.* 2003).

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According to some recent views, luteolysis might be regarded as an immune-mediated event leading to apoptosis (Tilly 1996), and in this context, the cytokines normally found also in CL of rabbits (Krusche *et al.* 2002) may exert a physiological role in luteal demise (Del Vecchio & Sutherland 1997, Penny 2000, Pate & Keyes 2001). Increasing evidence indicates that some cytokines may suppress or induce apoptosis (Lotem & Sachs 1999) as well as regulate NOS expression and activity in different ways, depending on cell type, cytokines and NOS isoform (Forstermann *et al.* 1995). Interestingly, a variety of genes for cytokines may be downstream targets of the short-lived secondary messenger NO (Torres & Forman 2000) produced by resident luteal cells or by the heterogeneous population of accessory immune cells present within the CL (Bagavandoss *et al.* 1990). At the same time, NO may promote the intracellular mechanisms of the apoptotic pathways or inhibit its activation (Kim *et al.* 1999).

Thus, to better characterise some of those factors that may regulate NOS activity, or respond to NO, the objectives of this study were to (i) examine whether the gene expression patterns for p53, whose product triggers apoptosis, and different cytokines, including macrophage chemoattractant protein-1 (MCP-1), interleukin (IL)-1β and IL-2, are regulated during the spontaneous luteolytic process in rabbits, (ii) investigate the relationship between both luteal eNOS and iNOS expression levels and NOS activity, and (iii) correlate NOS activity with luteal and plasma progesterone content. Since up to day 12 of pseudopregnancy NOS activity was not found to change (Boiti *et al.* 2003), for this *ex vivo* model, CL were collected throughout late luteal stages at days 13, 15, 18 and 22 of pseudopregnancy, across both functional and structural luteolysis.

Materials and Methods

Materials

Random hexamer primers, deoxyribonuclease I (DNAase I Amp Grade), RNAse H^- reverse transcriptase (Superscript II), *E. coli* RNase H and DNA ladders were obtained from Invitrogen (S. Giuliano Milanese, Milan, Italy). The reagent for isolation and purification of total RNA (TRIzol), Taq DNA polymerase (Platinum), RNAse-free tubes and RNAse-free water and deoxy-NTPs were also acquired from Invitrogen. Primers for 18S rRNA and corresponding competimers (QuantumRNA 18S Internal Standards) were obtained from Ambion (Austin, TX, USA), while those for each target NOS RNA were custom obtained from Invitrogen. Primers for mRNAs of p53 and cytokines MCP-1, IL-1β and IL-2 were supplied by Maxim Biothec Inc. (San Francisco, CA, USA). The kit for the assay of proteins was obtained from Bio-Rad Laboratories (Segrate, Milan, Italy). [2,3-^{3}H]L-arginine, having a specific activity of 30–40 Ci/mmol, and all other chemicals were from Sigma (St Louis, MO, USA). [1, 2, 6, 7-^{3}H] progesterone was purchased from Amersham Biosciences (Amersham Biosciences Ltd, Little Chalfont, Bucks, UK), while non-radioactive progesterone and antiserum came from Sigma. The NOS detectTM Assay Kit was purchased from Alexis Corp. (Läufelfingen, Switzerland). All other chemicals and reagents were pure grade and obtained locally. The following hormonal preparations were administered: gonadotrophin-releasing hormone (GnRH) analogue (Receptal; Hoechst-Roussel Vet, Italy) and pregnant mare serum gonadotrophin (PMSG) (Folligon; Intervet, Italy).

Animals, hormonal regimen and luteal tissue collection

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

Luteal tissue was obtained from unmated New Zealand White (HY/CR strain) rabbits (Charles River Italia, Lecco, Italy), 5 months of age, weighing 3.5–3.8 kg. The animals, caged individually in quarters of the University of Perugia Central Animal Facility, were maintained under controlled conditions of light (14 h light:10 h darkness) and temperature (18 °C), and provided with commercial rabbit chow and tap water freely. All rabbits received an i.m. injection of 20 IU of PMSG followed 3 days later by an i.m. injection of 0.8 μg GnRH to induce pseudopregnancy. The day of GnRH injection was designated day 0. This ovulation procedure was effective in generating an average of 14 or 15 CL per rabbit.

At days 13, 15, 18 and 22 of pseudopregnancy, five rabbits for each day were killed by cervical dislocation. CL were promptly excised from ovaries and rinsed with RNAse-free PBS. Non-luteal tissue was carefully dissected away with fine forceps under stereoscopic magnification. The CL harvested from each rabbit were immediately stored at −80 °C until processed for the enzymatic studies of total NOS activity, gene expression and progesterone content. From each rabbit, blood samples for progesterone assay were collected by venous puncture of the marginal ear vein, on the same days until immediately prior to killing. The samples were centrifuged at 3000 ***g*** for 15 min and plasma stored frozen until assayed for progesterone concentrations to assess the functional status of the ovarian CL. For the purpose of this work functional luteolysis was defined as a 50% decline of plasma progesterone from previous values, while complete luteolysis as the failure of CL to secrete progesterone so that blood levels decrease below 1.0 ng/ml, which are found in oestrous rabbits.

RNA extraction and reverse transcription

For each rabbit, total RNA was extracted from a pool of eight to ten CL, which were homogenised using Omni-μU (Analytical Control; Cinisello Balsamo, Milano, Italy) in 1 ml of the solution provided with TRIzol using the procedure as described by the manufacturer.

Concentration of total RNA (OD_{250}) and purity ($OD_{260/280}$, $OD_{260/230}$) was determined spectrophotometrically with a BioPhotometer (Eppendorf srl, Milan, Italy). The integrity of each sample was assessed by electrophoresis of an aliquot of 3 μg RNA in agarose formaldeyde gel using ethidium bromide staining. Genomic DNA contamination was prevented by treatment with deoxyribonuclease I (DNAase I Amp Grade) according to the manufacturer's instructions.

Five micrograms of total RNA (1 μg/μml) were reverse transcribed into cDNA in a 20 μl final reaction mixture in the presence of Superscript II reverse transcriptase following the manufacturer's procedure. The reverse transcription mixture consisted of 1 mmol/l d-NTPs and 100 ng random hexamer primers. The reaction was carried out for 50 min at 45 °C and was then inactivated by heating at 70 °C for 15 min. To remove the cRNA to the cDNA, 1 μl *E. coli* RNase H was added to the mixture and incubated at 37 °C for 30 min. Genomic DNA contamination was checked by carrying samples through PCR procedure without reverse transcriptase. The reverse transcription products were stored at − 20 °C.

Multiplex RT-PCR amplification

An aliquot (1.0 μl) of cDNA was used as a template for the subsequent semi-quantitative PCR amplification reaction containing two primer sets, one for the target gene (eNOS, iNOS, MCP-1, IL-1β, IL-2 and p53) and the other for a housekeeping gene (18S rRNA). Two sets of 18S housekeeping primers (324 and 489 bp) were used for the best discrimination of the target/housekeeping bands following gel electrophoresis.

The PCR reaction (25.0 μl) was performed with 0.2 μl Taq DNA Polymerase (5 U/μl), 1.0 μl dNTPs (10 mmol/l), 5.0 μl Taq buffer 10 ×, 3.0 μl mixed primers for each target gene (MCP-1, IL-1β, IL-2 and p53) and 18S housekeeping gene (489 bp) with competimers (2:8 v/v).

Sequences for custom primers or their code number (Maxim Biotech Inc.) were: MCP-1 (accession no. M57440, code no. CSM0003-MCP-1), product size 292 bp; IL-1β (accession no. M26295, code no. CSM0003-IL-1), product size 183 bp; IL-2 (accession no. AF068057, code no. CSM0003-IL2), product size 111 bp, forward 5′-AACTCAAACCTCTGGAGGAAGTGC-3′, reverse 5′-TCGATGCTGAGATGATGCTTTGACA-3′; p53 (accession no. 90592, code no. CSM004-p54), product size 300 bp; 18S (accession no. 10098): product size 489 bp, forward 5′-TCAAGAACGAAAGTCGGAGGTT-3′, reverse 5′-GGACATCTAAGGGCATCA-3′.

For the PCR of eNOS (or iNOS), 1.0 μl (10 μmol/l) of mixed primers for each target gene, both forward and reverse, were co-amplified with 18S housekeeping primers (324 bp), in the same master mix previously described. The primer sequences used were: eNOS (accession no. AF 287158), product size 485 bp, forward 5′-CAGTGTCCAACATGCTGCTGGAAATTG-3′, reverse 5′-TAAAGGTCTTCTTCCTGGTGATGCC-3′; iNOS (accession no. U85094), product size 537 bp, forward 5′-CAGGACCACACCCCCTCGGA-3′, reverse 5′-AGCCACATCCCGAGCCATGC-3′; 18S (accession no. M 10098), product size 324 bp, forward 5′-AGGAATTGACGGAAGGGCAC-3′, reverse 5′-GTGCAGCCCCGGACATCTAAG-3′.

The semi-quantitative PCR reactions were carried out as reported previously (Boiti *et al.* 2002). Preliminary experiments were carried out to establish the optimal ratio between 18S primers and their competimers, as well as the range of cycles. Between 30 and 40 cycles both target and 18S products were in a linear exponential phase of amplification (data not shown). To minimise errors, within each experiment, the target gene was co-amplified with housekeeping 18S primers at the same PCR cycle. The 18S rRNA is an ideal internal control for quantitative RNA analysis because its expression remains invariant across tissues and treatments, although its relatively high abundance makes it difficult to use in RT-PCR experiments because target mRNA species are by far less abundant. However, by mixing appropriate amounts of 18S primers with their competimers, primers modified only at their 3′ ends to block extension by DNA polymerase, the PCR amplification efficiency of 18S cDNA can be reduced to a level roughly similar to that of the gene under study. To summarise, amplification was performed on a thermal cycler (GeneAmp, PCR System; Perkin-Elmer Biosystem, Foster City, CA, USA). All PCR reactions consisted of a first denaturating cycle at 94 °C for 75 s, followed by an amplification profile of 35 reaction cycles with a first denaturating cycle at 94 °C for 15 s, followed by annealing at 60 °C for 30 s and extension at 72 °C for 45 s, and a final extension step at 72 °C for 10 min. Within each experiment and for each gene analysed, the complete set of samples was processed in parallel in a single PCR using aliquots of the same PCR master mix. Each set of determinations was performed in triplicate.

Analysis of amplification products

The amplified PCR-generated products (20 μl of 25 μl total reaction volume) were analysed by electrophoresis on 2% agarose gel using ethidium bromide staining. One product for each day of pseudopregnancy was electrophoresed on a single gel together with a negative control that contained no RNA and a standard DNA ladder. The gel images were acquired by using a Kodak DC290 digital camera. The background-corrected band intensities (absolute optical densities subtracted by the background levels from corresponding lanes) for each PCR product were quantified using Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA). To evaluate the temporal changes in relative levels of mRNAs, the band intensities for the target genes of interest obtained from each aliquot of PCR products were normalised against those of the housekeeping 18S mRNA co-amplified product in the same aliquot. Values

were expressed as arbitrary units of relative abundance of the specific target genes.

NOS activity determination

NOS activity was determined by monitoring the conversion of [^{3}H]L-arginine (Sigma) into [^{3}H]L-citrulline with a commercial NOS assay kit, according to the experimental protocol previously described (Boiti *et al.* 2000). Total NOS luteal activity of each rabbit was independently assayed in three CL randomly selected from the corresponding pool.

Progesterone assay

Progesterone concentrations in plasma samples and luteal tissue were determined by RIA, using specific antibody according to the procedure reported elsewhere (Boiti *et al.* 2003). Progesterone was extracted with ethyl ether from plasma or CL, after homogenisation in PBS in a Dounce homogeniser. The progesterone content of CL was expressed as ng/mg wet weight of luteal tissue. The assay sensitivity was 10 pg/ml; intra-assay and inter-assay coefficients of variations were 6 and 11% respectively.

Statistical analysis

All experiments were repeated three times and means ± S.E.M. of values (arbitrary expression units, progesterone concentrations and radioactivity) are given as a function of luteal age for the number of separate cases as reported in figure legends. The ratios of each PCR product for a target gene (MCP-1, IL-1β, IL-2, p53, eNOS and iNOS) normalised against the 18S co-amplified product were analysed by two-factor ANOVA (Sokal & Rohlf 1981) by taking into account the day of pseudopregnancy and gel as the two sources of variability, followed by a Newman–Keuls multi-comparison post-test.

Data relative to the time course of progesterone concentrations in plasma and luteal tissue and those of NOS activity during late pseudopregnancy and spontaneous luteolysis were analysed by ANOVA followed by Duncan's multiple range test (Duncan 1955). The relationship between total NOS luteal activities and progesterone levels was determined by correlation analysis. A value of $P < 0.05$ was considered significant.

Results

To study the time-dependent course of mRNA levels encoding the gene of interest during spontaneous luteolysis, samples of total RNA, extracted from CL of individual rabbits at different time points, were analysed by semi-quantitative multiple RT-PCR, using specific primers. In each PCR reaction, 18S rRNA was co-amplified and used as an internal standard for the calculation of the relative abundance of mRNA for the target genes. The expression of 18S rRNA in rabbit CL remained fairly constant, independently of the luteal phases. The corresponding base pair amplification products obtained using primers designed for each gene matched the expected sizes (see Figs 1–5).

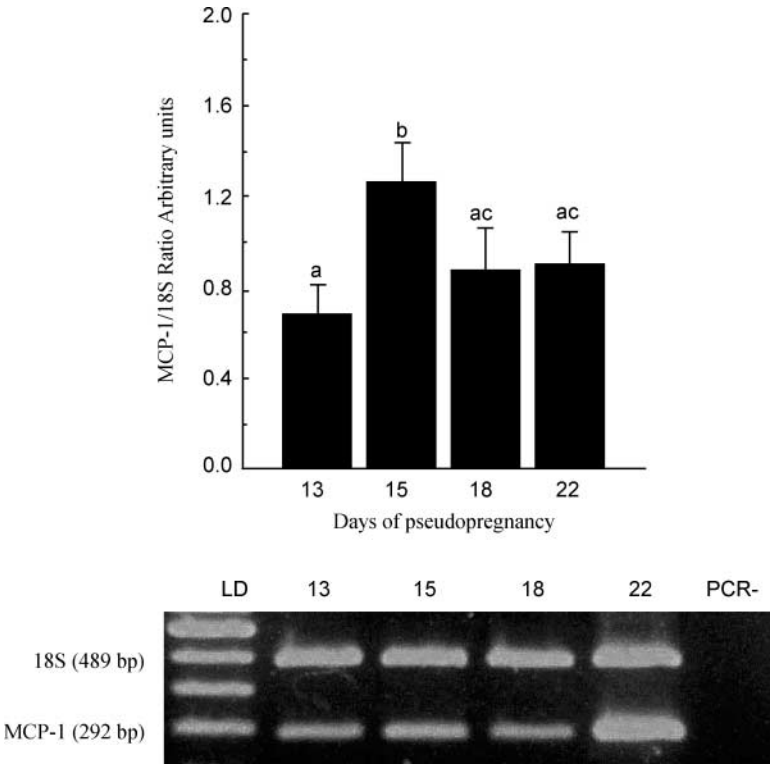

Figure 1 Expression profile of MCP-1 mRNA in CL of rabbits obtained at days 13, 15, 18 and 22 of pseudopregnancy. The lower panel shows a representative photograph of a 2% agarose ethidium bromide-stained gel used to analyse the PCR products. The sizes of the amplified products are shown on the left of the gel. LD is the kilobase DNA marker while lane PCR– represents a negative control of non-reverse-transcribed RNA submitted to PCR amplification. The other lanes identify the corresponding days of pseudopregnancy. The upper panel shows the data derived from densitometric analyses of the gels. For each luteal age, the values (means ± S.E.M.) combine the results from five different rabbits and are reported in arbitrary units of MCP-1 mRNA relative to that of 18S used as internal standard. Different letters above bars indicate a significantly different value ($P \leq 0.05$).

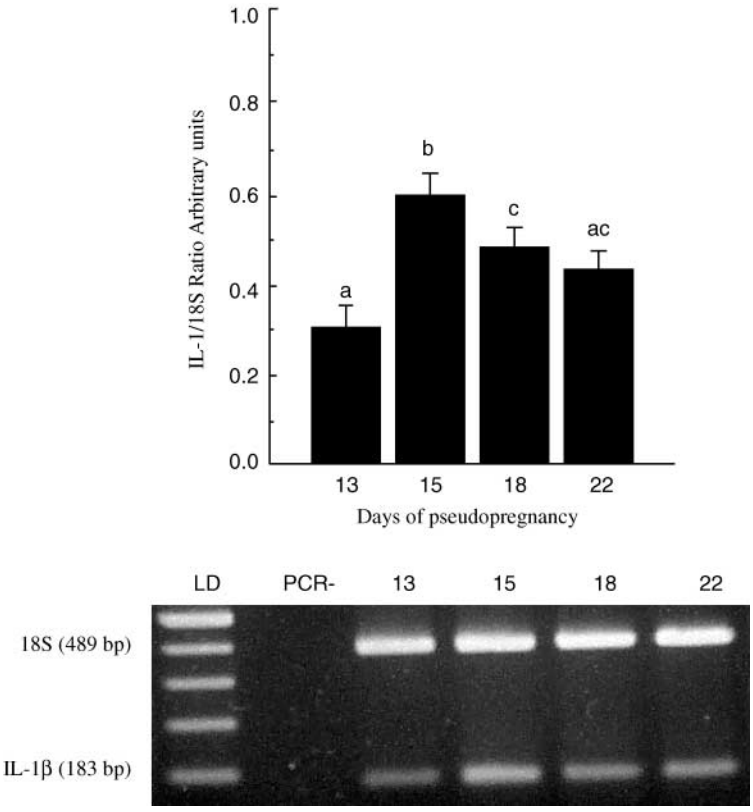

Figure 2 Expression pattern and levels of mRNA encoding IL-1β gene in CL of rabbits obtained at days 13, 15, 18 and 22 of pseudopregnancy during luteal regression. The PCR products were analysed by a 2% agarose ethidium bromide-stained gel. The lower panel shows a representative photograph of a gel. The sizes of the amplified products are shown on the left of the gel. LD is the kilobase DNA marker while lane PCR– represents a negative control of non-reverse-transcribed RNA submitted to PCR amplification. The other lanes identify the corresponding days of pseudopregnancy. The upper panel shows the data derived from densitometric analyses of the gels. For each luteal age, the values (means± S.E.M.) combine the results from five different rabbits and are reported in arbitrary units of IL-1β mRNA relative to that of 18S used as internal standard. Different letters above bars indicate a significantly different value ($P \leq 0.05$).

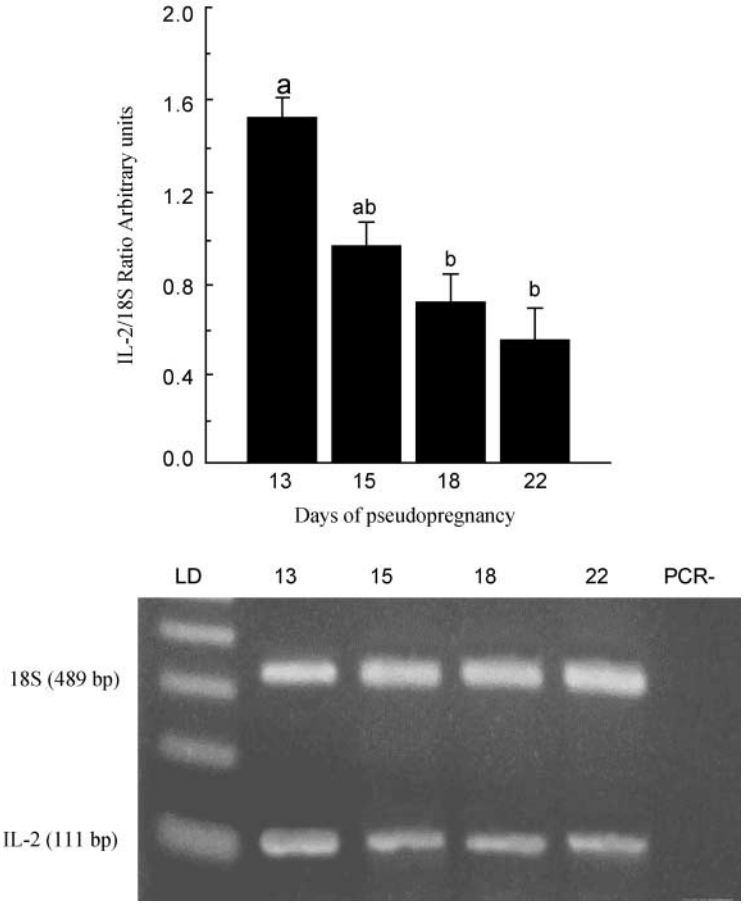

Figure 3 Expression profile and relative abundance of IL-2 mRNA in CL of rabbits obtained at days 13, 15, 18 and 22 of pseudopregnancy. The PCR products were analysed by a 2% agarose ethidium bromide-stained gel (lower panel). The sizes of the amplified products are shown on the left of the gel. LD is the kilobase DNA marker while lane PCR– represents a negative control of non-reverse-transcribed RNA submitted to PCR amplification. The other lanes identify the corresponding days of pseudopregnancy. The upper panel shows the data derived from densitometric analyses of the gels. For each luteal age, the values (means± S.E.M.) combine the results from five different rabbits and are reported in arbitrary units of IL-2 mRNA relative to that of 18S used as internal standard. Different letters above bars indicate a significantly different value ($P \leq 0.05$).

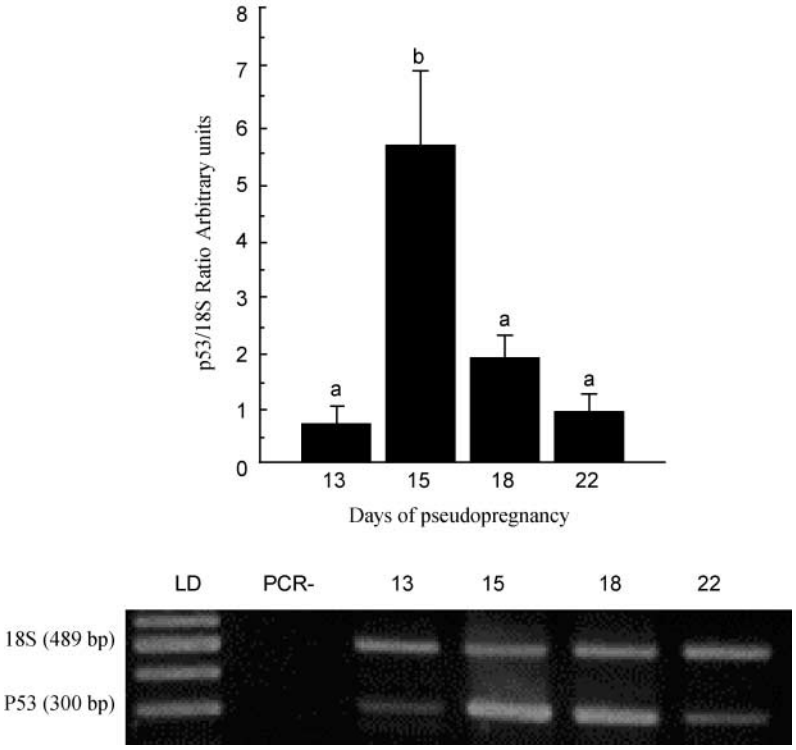

Figure 4 Expression profile and relative abundance of p53 mRNA in CL of rabbits obtained at days 13, 15, 18 and 22 of pseudopregnancy. The PCR products were analysed by a 2% agarose ethidium bromide-stained gel (lower panel). The sizes of the amplified products are shown on the left of the gel. LD is the kilobase DNA marker while lane PCR– represents a negative control of non-reverse-transcribed RNA submitted to PCR amplification. The other lanes identify the corresponding days of pseudopregnancy. The upper panel shows the data derived from densitometric analyses of the gels. For each luteal age, the values (means± S.E.M.) combine the results from five different rabbits and are reported in arbitrary units of p53 mRNA relative to that of 18S used as internal standard. Different letters above bars indicate a significantly different value ($P \leq 0.05$).

Gene expression of mRNA for luteal cytokines and p53

MCP-1 mRNA levels rose 2-fold ($P \leq 0.01$) from day 13 to 15 of pseudopregnancy, but then dropped ($P \leq 0.05$) by day 18 onward (Fig. 1). The gene transcript for IL-1β exhibited a similar pattern (Fig. 2) with a peak in its expression at day 15 of pseudopregnancy when its relative abundance was 90% higher ($P \leq 0.01$) than that found at day 13. From day 18 of pseudopregnancy onward, IL-1β mRNA levels gradually fell. IL-2 relative mRNA abundance (Fig. 3) was higher ($P \leq 0.05$) on day 13 and then gradually declined during the course of luteal regression.

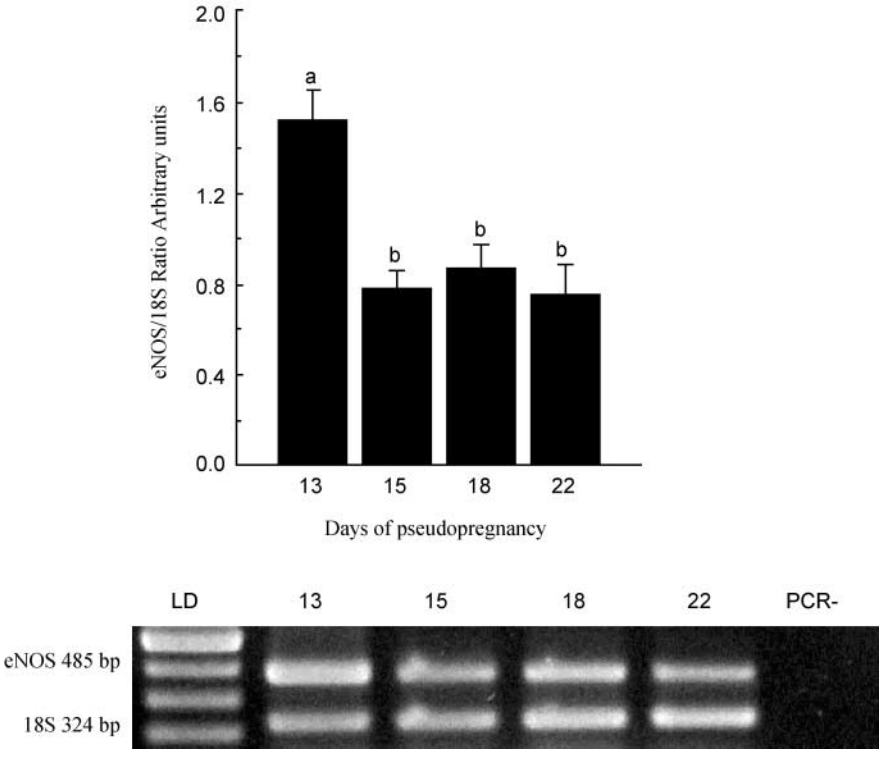

Figure 5 Gene expression patterns of eNOS mRNA in CL of rabbits harvested at days 13, 15, 18 and 22 of pseudopregnancy. The lower panel is a representative photograph of a typical 2% agarose, ethidium bromide-stained gel, showing the presence of the expected 485 bp products yielded after RT-PCR using primers for target eNOS and 18S. Lane LD is the kilobase DNA marker, lane PCR– represents a negative control of non-reverse-transcribed RNA submitted to PCR amplification, while the other lanes identify the corresponding days of pseudopregnancy. The upper panel summarises the data derived from densitometric analyses of the gels from the experiments of natural luteolysis. The values (means± S.E.M.) combine the results from five different rabbits for each luteal age and are reported in arbitrary units of eNOS mRNA relative to that of 18S used as internal standard. Different letters above bars indicate a significantly different value ($P \leq 0.05$).

The p53 mRNA levels markedly rose ($P \leq 0.01$) 8-fold from day 13 to 15 of pseudopregnancy and then gradually declined from day 18 onward ($P \leq 0.05$) to the same levels found at day 13, before the onset of functional luteolysis (Fig. 4).

Gene expression of NOS isoforms mRNA

In the course of luteal regression, eNOS mRNA levels in CL decreased by almost 40% ($P \leq 0.05$) from day 13 to 15, and then remained unchanged throughout the late luteal stages in both day 18 and 22 CL (Fig. 5). During the same time interval, iNOS mRNA was barely detectable, ranging between 0.26 ± 0.12 and 0.15 ± 0.04 (arbitrary units), but no clear patterns in its steady state levels were observable (data not shown).

Total NOS activity and progesterone

During the late luteal phase of pseudopregnancy, total NOS activity progressively increased ($P < 0.05$) 2-fold from day 13 to 18 CL and then markedly dropped to the lowest levels in day 22 CL (Fig. 6).

Luteal progesterone content decreased with the ageing of CL during late luteal phases, from 411 pg/mg wet weight on day 13 to 17 pg/mg on day 22 (Fig. 7A). The changes in NOS total activity were inversely correlated with decreases in luteal progesterone content on days 13, 15 and 18 of pseudopregnancy ($r = -0.92$, $P \leq 0.01$).

Similarly, plasma progesterone concentrations, used as a marker of luteal functionality, markedly dropped ($P \leq 0.01$) in rabbits from day 13 to 15 of pseudopregnancy (12.3 ± 1.5 and 1.7 ± 0.3 ng/ml respectively) and then gradually decreased to the lowest values (0.4 ± 0.2 ng/ml) found at day 22 (Fig. 7B).

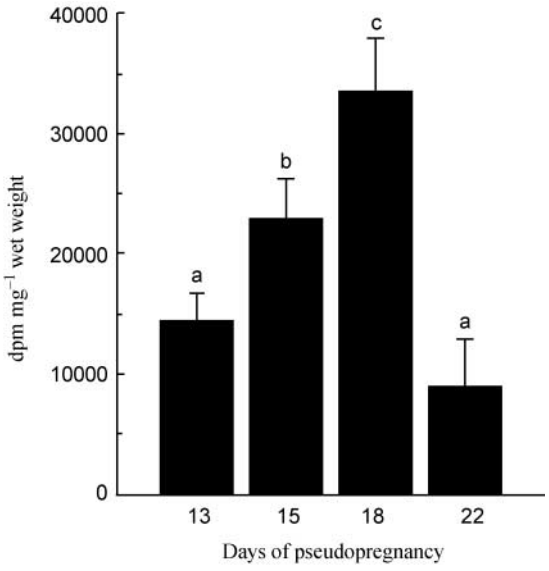

Figure 6 Changes in total NOS activity from day 13 to 22 of pseudopregnancy. Results refer to mg of wet tissue and represent means ± S.E.M. of five combined values for each data point. Different letters above bars indicate a significantly different value ($P \leq 0.05$).

Discussion

The main aim of this study was to verify whether the activity of NOS and the levels of genes encoding NOS isoforms, both eNOS and iNOS, and cytokines MCP-1, IL-1β and IL-2 as well as p53 were dynamically regulated in CL of rabbits in the course of spontaneous luteolysis. In the present study, we have demonstrated for the first time that changes in luteal p53 gene transcription during spontaneous luteolysis are closely coupled with the up-regulation of luteal NOS total activity and are coincident with progesterone decline. Moreover, the expression patterns of MCP-1, IL-1β and IL-2 suggest, although indirectly, that these cytokines may be involved in the regulation of NOS expression and activity in CL, thus playing a role in their regression.

In the present study, the increased gene expression for luteal cytokines MCP-1 and IL-1β detected at day 15 of pseudopregnancy may reflect the increased traffic of different immune cell types, including macrophages associated with luteal demise (Bagavandoss *et al.* 1990, Naftalin *et al.* 1997, Krusche *et al.* 2002). In the rabbit CL, according to Bagavandoss *et al.* (1998), T lymphocytes precede the presence of macrophages. The influx of these T cells, therefore, may be responsible for the higher expression of IL-2 mRNA found in rabbit CL at day 13 of pseudopregnancy compared with older, regressing CL. In this study, the temporal expression patterns of mRNAs for the cytokines here examined were different from those recently reported by Krusche *et al.* (2002) during functional and structural regression. However, besides the different hormonal protocols used for inducing ovulation, the diverse analytical approaches to measure mRNA abundance may partially explain this discrepancy. In our study, in fact, the relative gene expression levels were quantified with greater accuracy using the multiplex RT-PCR technique, by co-amplifying each target gene with 18S rRNA as internal standard.

A 2- to 3-fold rise in NOS activity occurred at days 15 and 18 of pseudopregnancy during the transition of the CL from the late-luteal stage to functionally regressed CL, as evidenced by the concomitant fall of both luteal progesterone content and plasma concentrations towards basal values. In the present study we have not attempted to differentiate the specific contribution to total bio-activity between the two isoforms. Therefore, it remains to be verified whether the increased production of NO observed during the course of luteolysis derives from eNOS or mainly from iNOS, as found in rats (Motta & Gimeno 1997). Whereas eNOS enzyme does not depend upon new protein synthesis to rapidly synthesise and release NO, iNOS is mainly regulated at the expression level, requiring new protein synthesis to catalyse the formation

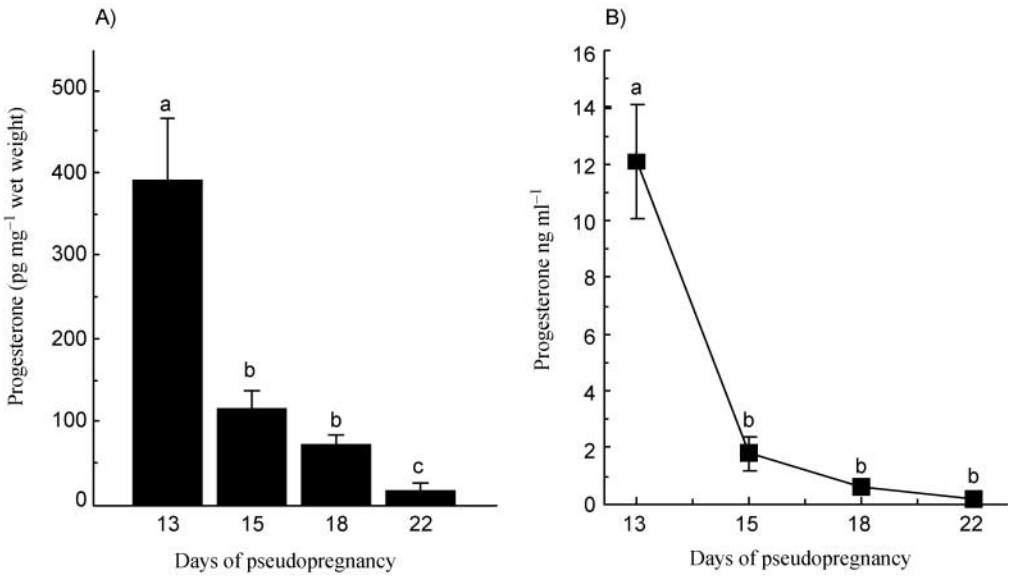

Figure 7 (A) Luteal progesterone content and (B) progesterone levels in blood throughout late luteal stages, from day 13 to 22 of pseudopregnancy. Results for luteal progesterone content refer to mg of wet tissue, while those for plasma progesterone concentrations refer to ng/ml. Values are means ± S.E.M. for five animals per group. Different letters indicate a significantly different value ($P \leq 0.01$).

of NO, which is also produced in larger amounts and for a longer period of time compared with the constitutive isoforms (Forstermann *et al.* 1995).

The marked down-regulation of luteal eNOS mRNA from day 15 of pseudopregnancy onward suggests a lowered RNA synthesis of the gene or, alternatively, an increased eNOS mRNA destabilisation, although it may simply reflect the dynamic changes of the heterogeneous luteal cell population occurring in the course of the CL life span (Nicosia *et al.* 1995). By contrast, the mRNA levels encoding iNOS were barely detectable and no age-related trend was traced throughout late luteal stages of pseudopregnancy and during both phases of luteal regression. In rabbit CL, immunohistochemical staining revealed that iNOS is localised only in a few immune cells (Boiti *et al.* 2002) and thus the low levels of iNOS mRNA may be partially explained by the variable presence of a small number of activated macrophages.

Recent evidences based on *in vitro* experiments indicate that some of the cytokines here examined may also regulate NOS expression and activity depending on cell type, cytokine and NOS isoform (Gross & Levi 1992). IL-2 was shown to promote eNOS mRNA (Juretic *et al.* 1995), whereas IL-1β augmented NOS activity in ovarian dispersate of rats (Estevez *et al.* 2002). At the same time, IL-1β induces iNOS gene expression by activating the transduction factor nuclear factor-κB (Forstermann *et al.* 1995), but other ILs can inhibit its expression (Bogdan *et al.* 1994).

Besides cytokines, a large variety of agents including progesterone (Miller *et al.* 1996), cAMP-elevating compounds (Mustafa & Olson 1998) and NO itself (Taylor *et al.* 1997) have been shown to inhibit cytokine-induced iNOS transcription in different cell lines. Thus, it is not clear at this time whether the up-regulation in luteal NOS activity during spontaneous luteolysis is associated with an induction of eNOS and/or iNOS genes and new protein synthesis around day 14 of pseudopregnancy, with increased substrate supply, or with other post-translational mechanisms (Boiti *et al.* 2001). Nevertheless, it is important to note that, despite the different time-scale employed to examine the two luteolytic processes, similar findings were observed also in PGF2α-induced luteolysis when NOS activity markedly rose within 6 h after PGF2α challenge and remained high throughout the following 48 h, whereas eNOS gene transcription decreased by 40% and that for iNOS remained unaffected (Boiti *et al.* 2003).

In the present study, the expression levels for p53 mRNA were markedly enhanced in day 15 CL. This finding documents that the apoptotic pathway is already activated, on a molecular level, at this luteal stage concurrently with functional luteolysis and precedes the morphological changes typical of cell death observed in CL at day 18 of pseudopregnancy (Nicosia *et al.* 1995). It is now well recognised that the p53 gene can induce apoptosis by modifying the balance between the anti- and pro-apoptotic related proteins, such as Bcl-2 and Bax, controlled by several genes (Reed 1998). The switch in the relative abundance of this gene appears coincidental with the marked increase in total NOS activity. This finding suggests that *in vivo* exposure to increased levels of NO, NO-derived peroxynitrite and/or other reactive oxygen species (ROSs) generated during luteolysis, may modulate luteal apoptosis through up-regulation of the p53 gene as already demonstrated by *in vitro* studies on luteal cells treated with H_2O_2 (Nakamura & Sakamoto 2001). The transient increase of p53 transcription at day 15 of

pseudopregnancy, however, is also coincident with progesterone decline. Thus, the marked up-regulation of p53 gene in CL may be triggered by removal of the progesterone blockade, which exerts a protective action on luteal cell survival and opposes functional regression of CL (Goyeneche *et al.* 2003).

Taken together, these results give strength to the hypothesis that the NO/NOS system is involved in CL regression *in vivo* as already documented for luteolysis induced by exogenous administration of PGF2α (Boiti *et al.* 2003). In the ovary and luteal tissue, endogenous NO, besides regulating the activity of key enzymes such as P450/scc, ADP-ribosylating enzymes, dehydrogenases, phosphatases and other transcription factors (Gow & Ischiropoulos 2001), may also inhibit 17β-oestradiol synthesis (Yamauchi *et al.* 1997), which exerts a well-known luteotrophic action in rabbits (Holt 1989), or stimulate luteal PGF2α release through the activation of cyclooxygenase (Estevez *et al.* 2002). Within the CL, overproduction of NO during luteolysis may target nitration and oxidation in specific luteal cellular compartments thereby affecting specific protein function as well as causing lipid peroxidation (Aten *et al.* 1998, Motta *et al.* 2001) or DNA damage (Beckman *et al.* 1990). Moreover, high concentrations of NO may promote apoptosis (Kim *et al.* 1999).

In summary, the present data are consistent with the hypothesis that spontaneous luteolysis in rabbit CL is evoked through an up-regulation of NOS activity and increased release of NO and ROSs as already observed in luteal regression induced by exogenous PGF2α. Probably, the enhanced NOS activity is mediated by locally produced factors, including cytokines secreted by resident cells or recruited immune cells leading to apoptosis. It must be stressed, however, that a number of details of this proposed pathway are still unknown, including the exact sequence of events which, directly or indirectly, cause the up-regulation of NOS activity, and regulate the gene expression of NOS isoforms and those for cytokines MCP-1, IL-1β and IL-2 and p53. The up-regulation of MCP-1, IL-1β and p53 transcripts at day 15 post-ovulation is inversely correlated with the decrease in both plasma and luteal progesterone levels, suggesting that the removal of progesterone blockade may increase their transcription. Also the actions of these cytokines on luteal function, as well as their physiological relevance and the cross talk between immune cells and luteal cells remain to be better investigated. There is little doubt that complex interplay between different cytokines, due to their multiple and redundant actions, is at work in the CL during regression. Also, the signal interactions and the balance between pro- and anti-apoptotic proteins demand further investigation.

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