Regulation of nitric oxide synthase isoforms and role of nitric oxide during prostaglandin F_{2α}-induced luteolysis in rabbits

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Total activity of nitric oxide synthase (NOS) and the gene expression of both endothelial NOS (eNOS) and inducible NOS (iNOS) isoforms in corpora lutea of pseudopregnant rabbits were examined during prostaglandin F_{2α} (PGF_{2α})-induced luteolysis. Corpora lutea were collected at 0, 6, 12, 24 and 48 h after an injection of PGF_{2α} at day 9 of pseudopregnancy. At 12 h after PGF_{2α} administration, luteal mRNA encoding eNOS decreased ($P \leq 0.05$) by 40% and remained low throughout the subsequent 36 h, whereas eNOS protein increased ($P \leq 0.05$) two- to threefold. By contrast, expression of mRNA encoding iNOS was poor and remained fairly constant, but transcription increased eightfold ($P \leq 0.01$) within 6 h after PGF_{2α} treatment and then decreased to values similar to those of controls. Total NOS activity increased twofold ($P \leq 0.01$) at 6 h after treatment and remained high thereafter,

whereas progesterone concentrations in explanted corpora lutea decreased ($P \leq 0.01$) from 302.4 ± 42.3 pg mg⁻¹ at day 9 to 58.6 ± 8.3 at 48 h later, and peripheral plasma concentrations of progesterone declined too. Long-term administration of N^ω-nitro-L-arginine methyl ester (0.6 g l⁻¹ per os) from day 2 of pseudopregnancy onward partially blocked the luteolytic action of PGF_{2α} administered at day 9 of pseudopregnancy. In nitric oxide (NO)-deficient rabbits, progesterone concentrations remained higher ($P \leq 0.01$) than in controls at 24–48 h after PGF_{2α} administration (4.5 to 3.2 ng ml⁻¹, respectively). These data are the first to characterize NOS activity. The time course of expression of eNOS and iNOS in rabbit corpora lutea during PGF_{2α}-induced luteolysis gives additional support to a physiological role of NO in the regulation of regression of corpora lutea in rabbits.

Introduction

Luteolysis is a complex and streamlined process that involves not only functional but also structural changes that lead to the complete demise of the corpus luteum (McCracken *et al.*, 2000). In rabbits, which are reflex ovulators, the luteolytic mechanisms begin either after infertile mating to reduce the duration of pseudopregnancy by shortening the lifespan of unnecessary corpora lutea or at the end of gestation (Scott and Rennie, 1970; Carlson and Gole, 1978). As in most other mammalian species, luteolysis is driven by prostaglandin F_{2α} (PGF_{2α}), which has been identified as the main luteolysin factor of uterine origin (O'Grady *et al.*, 1972; Keyes and Bullock, 1974; Lytton and Poyser, 1982). However, besides species differences, not all the events involved in this process are well understood, and a set of hypotheses has been proposed to explain luteolysis (Niswender *et al.*, 2000).

An increasing body of evidence supports the concept that nitric oxide (NO), a novel effector and signalling molecule produced from L-arginine (Moncada *et al.*, 1991) by the action of different nitric oxide synthases (NOS), may have a significant role in the control of

several key functions in reproductive physiology (Dixit and Parvizi, 2001). Gobbetti *et al.* (1999) reported that NO exerted a regulatory role on progesterone release by corpora lutea of pseudopregnant rabbits *in vitro*. It was hypothesized that the inhibitory and luteolytic effects on progesterone triggered by PGF_{2α} *in vitro* are controlled, at least in part, by the NO–NOS system activating the phospholipase C–protein kinase C pathway (Boiti *et al.*, 2000). Boiti *et al.* (2002) reported the expression patterns of both constitutive endothelial NOS (eNOS) and inducible NOS (iNOS) and their cellular localization in corpora lutea of pseudopregnant rabbits during different luteal stages.

NO has been proposed as a potential autocrine regulator of luteal steroidogenesis (Van Voorhis *et al.*, 1994; Olson *et al.*, 1996; Hesla *et al.*, 1997; Yamauchi *et al.*, 1997; Vega *et al.*, 1998; Faletti *et al.*, 1999; Nakamura *et al.*, 1999; Friden *et al.*, 2000; Jaroszewski and Hansel, 2000). Therefore, the aim of this study was to verify whether NO is effectively implicated in the luteolytic mechanisms *in vivo*. Experiments were designed to investigate (i) the relationship between luteal NOS activity and progesterone content in both corpus luteum and peripheral blood, and (ii) the regulation of both eNOS and iNOS luteal genes and proteins

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during PGF_{2α}-induced luteolysis in rabbits. The role of the NO–NOS system *in vivo* was evaluated further to investigate whether chronic administration of L-NAME, a NOS inhibitor, blocked the functional luteolysis triggered by PGF_{2α}.

In all experiments, PGF_{2α} was injected at day 9 of pseudopregnancy when rabbits have been shown to be completely responsive to this compound (Boiti *et al.*, 1998).

Materials and Methods

Materials

The monoclonal mouse anti-human eNOS antibody was purchased from Transduction Laboratories (Lexington, KY), as were human endothelium-derived eNOS and mouse macrophage-derived iNOS lysates. Anti-mouse iNOS antibody was obtained from R&D Systems Inc. (Minneapolis, MN). The biotinylated secondary antibody and avidin–biotin peroxidase reagents for the detection system (Vectastain, ABC reagent, Vector Elite Kit), together with DAB chromogenic substrate kit, were all obtained from Vector Laboratories Inc. (Burlingame, CA).

Random hexamer primers, deoxyribonuclease I (DNAase I Amp Grade), RNase H[−] reverse transcriptase (Superscript II) and DNA ladders were obtained from Invitrogen Italia (Giuliano Milanese, Milan). The reagent for isolation and purification of total RNA (TRIzol), Taq DNA polymerase (Platinum), RNase-free tubes, RNase-free water and deoxy-NTPs were also acquired from Invitrogen. Primers for 18S rRNA (QuantumRNA 18S Internal Standards) were obtained from Ambion (Austin, TX), whereas those for each target eNOS and iNOS RNA were custom made by Invitrogen. The kit for the assay of proteins was obtained from Bio-Rad Laboratories (Segrate, Milan). [2,3-³H]-L-arginine, which has a specific activity of 30–40 Ci mmol^{−1} was obtained from Sigma (St Louis, MO). [1,2,6,7-³H]progesterone was purchased from Amersham Biosciences (Amersham Biosciences Ltd, Little Chalfont), whereas non-radioactive progesterone and antiserum were from Sigma. The NOS detectTM assay kit was purchased from Alexis Corp. (Läufelfingen). N^ω-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NOS, came from Sigma. All other chemicals and reagents were pure grade and obtained locally. The following hormonal preparations were administered: GnRH analogue (Receptal, Hoechst-Roussel Vet, Milan), equine chorionic gonadotrophin (eCG) (Folligon, Intervet, Milan) alfaprostol (Gabbrostim, Centralvet, Milan), a PGF_{2α} analogue, and natural PGF_{2α} (Sincro Bovis, Gellini, Latina).

Animals and *in vivo* stimulation protocols

Sexually mature New Zealand white (HY/CR strain) female rabbits (Charles River Italia, Lecco) were main-

tained in the University of Perugia Central Animal Facility under controlled conditions of light (14 h light:10 h dark) and temperature (18°C) for a minimum of 3 weeks before the experimental procedure. The animals were caged individually and were provided with commercial rabbit chow and tap water *ad libitum*.

All rabbits received an i.m. injection of 20 iu eCG 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 corpora lutea per rabbit.

Experiment 1

On day 9 of pseudopregnancy, rabbits received an i.m. injection of 200 µg alfaprostol (*n* = 15) or 200 µg saline (*n* = 15). Three rabbits from each group were killed by cervical dislocation immediately after PGF_{2α} or saline injection (time 0) and the remainder at 6, 12, 24 or 48 h after treatment. Corpora lutea were immediately excised from ovaries and rinsed with RNase-free PBS. Non-luteal tissue was carefully dissected with fine forceps under stereoscopic magnification. The corpora lutea harvested from each rabbit were immediately frozen in liquid nitrogen and stored at −196°C until processed for the enzymatic studies of total NOS activity, expression of both eNOS and iNOS, and progesterone content. From each rabbit, two blood samples were collected by venous puncture of the marginal ear vein, one just before treatment and the other immediately before the rabbits were killed. The samples collected in EDTA vacutainers were centrifuged at 3000 *g* for 15 min and plasma was frozen until assayed for progesterone to assess the functional status of the ovarian corpus luteum.

Experiment 2

From day 2 to day 9 of pseudopregnancy, rabbits were provided with drinking water containing the NOS inhibitor L-NAME (0.6 g l^{−1}), whereas other does were left untreated during the same period and served as controls. On day 9 of pseudopregnancy, L-NAME-treated rabbits received i.m. injections of 2.5 mg natural PGF_{2α} (*n* = 6) or 2.5 mg saline (*n* = 6), whereas control rabbits (*n* = 6) received PGF_{2α} at the same dose.

Blood samples were collected from each animal by venepuncture of the marginal ear vein at days 0, 3, 7 and 9 of pseudopregnancy, and subsequently by catheterization of the central ear vein at 2, 4, 6, 8, 24 and 48 h after PGF_{2α} or saline treatments. The samples were processed as described above.

RT-PCR amplification of eNOS and iNOS

For each rabbit, total RNA was extracted from a pool of eight corpora lutea. Corpora lutea were homogenized

in 1 ml of the solution provided with TRIzol using the procedure described by the manufacturer. Genomic DNA contamination was prevented by treatment with deoxyribonuclease I according to the instructions of the manufacturer. Total RNA (5 μg) was reverse transcribed into cDNA in a 20 μl final reaction mixture in the presence of Superscript II reverse transcriptase following the procedure of the manufacturer. The reaction was carried out in the RT buffer supplied with the enzyme. The reverse transcription mixture consisted of 1 mmol d-NTPs l^{-1} and 1 ng random hexamer primers and was carried out for 50 min at 45°C followed by an inactivation step (94°C, 10 min). The resulting cDNA (1 μl) was used as a template for the subsequent semi-quantitative PCR amplification reaction containing two primer sets, one for eNOS (or iNOS) gene and the other for 18S rRNA housekeeping gene. The primers designed for target genes were based on published sequences for rabbit iNOS (GenBank, accession no U85094) and eNOS (Boiti *et al.*, 2002) and custom made by Invitrogen.

Primers for eNOS were sense, 5'-CAGTGTCCA-ACATGCTGCTGGAAATTG-3' (F1), and antisense, 5'-TAAAGGTCTTCTCCTGGTGATGCC-3' (R1). Primers for iNOS were sense, 5'-CAGGACCACACCCCTCGGA-3' (F2), and antisense, 5'-AGCCACATCCCG-AGCCA-TGC-3' (R2). The semi-quantitative PCR reactions were carried out as reported by Boiti *et al.* (2002). Within each experiment and for each gene analysed, the complete set of samples was processed in parallel in a single PCR reaction using aliquots of the same PCR master mix. Each set of determinations was performed in triplicate. Preliminary experiments were carried out to establish the optimal ratio between 18S primers and their competitors, as well as the range of cycles over which both target and 18S products were in a linear exponential phase of amplification. The amplified products (20 μl of 50 μl total reaction volume) were analysed by electrophoresis on 2% agarose gel using ethidium bromide staining. One product for each time point was subjected to electrophoresis on a single gel together with a negative control that contained no RNA and standard DNA ladder. The images of gels were acquired using a Kodak DC290 digital camera. PCR products were quantified using Quantity One software (Bio-Rad Laboratories, Hercules, CA). The band intensities for the target genes of interest obtained from each aliquot of PCR products were normalized against those of the housekeeping 18S mRNA, a co-amplified product in that aliquot to evaluate the temporal changes in relative amounts of eNOS or iNOS mRNAs. Values were expressed as arbitrary units of relative abundance of the specific target genes.

Protein extraction and western blot analysis

Luteal proteins were extracted from corpora lutea of each rabbit as described by Boiti *et al.* (2002). Briefly,

the protein contents in the resulting supernatant samples were estimated using the Bio-Rad protein assay kit. Equivalent amounts of protein (10 μg) were separated by electrophoresis by discontinuous 10% SDS–PAGE with 4% stacking gel for 1 h at 200 V after which the proteins were transferred on to a nitrocellulose membrane (Protran, Schleicher and Schuell, Dassel) for 1 h at 100 V. After transfer, membranes were washed and blocked with 1% (w/v) casein in Tris-buffered saline (TBS-C; 20 mmol Tris–HCl l^{-1} , pH 7.4, 137 mmol NaCl l^{-1}). Blots were incubated overnight at 4°C with anti-eNOS (1:2000) or anti-iNOS (1:1000) antibodies, and subsequently with anti-mouse IgG–biotin conjugated secondary antibody for 30 min at 37°C with gentle agitation. All antibody incubations and washings were performed in TBS-C. Immunoreactive bands were developed using DAB substrate for peroxidase after the addition of the avidin–biotin complex. Mouse macrophage iNOS and human eNOS lysates were used as positive controls. After the acquisition of blot images with a HP scanner, the absorbances of the bands were quantified using Quantity One software. Each specific band signal was corrected by subtracting the background levels taken within the same lane and reported as arbitrary units.

Determination of NOS activity

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

Progesterone assay

Progesterone concentrations in plasma samples and luteal tissue were determined by radioimmunoassay, using specific antibody according to the procedure reported by Gobbetti *et al.* (1992). Progesterone was extracted with ethyl ether from plasma or corpus luteum after homogenization in PBS in a Dounce homogenizer. The progesterone content of corpus luteum was expressed as ng mg^{-1} of luteal tissue wet mass. The assay sensitivity was 10 pg ml^{-1} , and the intra-assay and interassay coefficients of variation were 6 and 11%, respectively. Complete functional luteolysis was interpreted as a decline of plasma progesterone concentrations to values of ≤ 1.0 ng ml^{-1} , which are observed in oestrous rabbits.

Statistical analysis

All experiments were repeated at least three times. Results are expressed as means $\pm$ SEM for the number of separate experiments. The ratios of each PCR product for

Fig. 1. (a) Expression patterns of mRNA encoding endothelial nitric oxide synthase (eNOS) in corpora lutea of rabbits examined during prostaglandin F_{2α} (PGF_{2α})-induced luteolysis as determined by multiplex RT-PCR. Corpora lutea were collected at 0, 6, 12, 24 and 48 h after injection of PGF_{2α} analogue, alfaprostol (□), or saline (■) administered at day 9 of pseudopregnancy. Total RNA was extracted from pooled corpora lutea of individual rabbits and was subsequently reverse transcribed and amplified. (b,c) Representative photographs of typical 2% agarose gels stained with ethidium bromide, showing the presence of the expected amplification products yielded after RT-PCR using primers for target eNOS (485 bp) and 18S (324 bp). In both gels, LD is the kilobase DNA marker and PCR- represents a negative control of non-reverse-transcribed RNA submitted to PCR amplification. The other lanes identify the corresponding hours after (b) saline or (c) PGF_{2α} injection. (a) Data derived from densitometric analyses of all the gels examined. The values (means ± SEM) combine the results from three different rabbits for each time point and are reported in arbitrary units of eNOS mRNA relative to that of 18S used as internal standard. Bars with a different letter are significantly different ($P \leq 0.05$) both within and between groups compared with zero time.

target gene normalized against 18S co-amplified product were analysed by two factor ANOVA (luteolytic treatment or day of pseudopregnancy and gel as the two sources of

variability) and Newman-Keul's multi-comparison post-test.

Data relative to overall treatment effects during PGF_{2α}-induced luteolysis were analysed by ANOVA (Sokal and Rohlf, 1981) followed by Duncan's multiple range test (Duncan, 1955). The relationship between total NOS luteal activities and progesterone concentrations was determined by correlation analysis. A value of $P < 0.05$ was considered significant.

Results

Gene expression of mRNA encoding NOS isoforms

Samples of total RNA extracted from corpora lutea of individual rabbits at different time points were analysed by semi-quantitative multiple RT-PCR, using primers specific for rabbit eNOS (Fig. 1) and iNOS (Fig. 2). In each PCR reaction, 18S rRNA was co-amplified and used as the internal standard for the calculation of the relative abundance of mRNA for the target genes. Expression of 18S rRNA in rabbit corpora lutea was not affected by PGF_{2α} treatment. The relative amount of mRNA encoding eNOS decreased significantly ($P \leq 0.05$) by 40% within 12 h of PGF_{2α} injection administered at day 9 of pseudopregnancy. mRNA encoding eNOS was low ($P \leq 0.05$) compared with controls thereafter (Fig. 1). In contrast, expression of iNOS mRNA was poor compared with expression of eNOS mRNA and did not change during the 2 days after PGF_{2α} treatment when compared with synchronous corpora lutea obtained from untreated control rabbits (Fig. 2).

Luteal eNOS and iNOS proteins

eNOS proteins were detected in the corpora lutea of all rabbits examined (Fig. 3). The immunoblot for eNOS showed two bands very close together of the expected 130–135 kDa and bands of lower molecular mass, approximately 70–75 kDa, which might represent fragments of eNOS. There was a two- to threefold increase in the abundance of eNOS protein ($P \leq 0.05$) at 12–48 h after PGF_{2α} treatment (Fig. 3) compared with basal, pre-treatment amounts or to amounts found in synchronous corpora lutea collected from control rabbits. iNOS proteins of the expected 130 kDa were also detected in all corpora lutea examined (Fig. 4). iNOS protein increased ($P \leq 0.01$) almost eightfold within the first 6 h after PGF_{2α} injection, but thereafter decreased to amounts similar to those of controls (Fig 4). Western blot analysis probing of iNOS in luteal tissue detected bands below that of the expected molecular mass. These proteins of approximately 80 and 72 kDa are considered to represent the homodimeric form and fragments of the enzyme, respectively (Jablonka-Shariff and Olson, 1997; Coers *et al.*, 1998; Watanabe *et al.*, 1998).

Fig. 2. (a) Expression pattern of mRNA encoding inducible nitric oxide synthase (iNOS) in corpora lutea of rabbits examined during prostaglandin $\text{F}_{2\alpha}$ ($\text{PGF}_{2\alpha}$)-induced luteolysis as determined by multiplex RT-PCR. Corpora lutea were collected at 0, 6, 12, 24 and 48 h after injection of $\text{PGF}_{2\alpha}$ analogue, alfaprostol (□), or saline (■) at day 9 of pseudopregnancy. Total RNA was extracted from pooled corpora lutea of individual rabbits, and was subsequently reverse transcribed and amplified. (b,c) Representative photographs of typical 2% agarose gels stained with ethidium bromide, showing the presence of the expected products yielded after RT-PCR using primers for target iNOS (537 bp) and 18S (324 bp). In both gels, LD is the kilobase DNA marker and PCR- represents a negative control of non-reverse-transcribed RNA submitted to PCR amplification. The other lanes identify the corresponding hours after (b) saline or (c) $\text{PGF}_{2\alpha}$ injection. (a) Data derived from densitometric analyses. The values (mean $\pm$ SEM) combine the results from three different rabbits for each time point and are reported in arbitrary units of iNOS mRNA relative to that of 18S used as internal standard.

Total NOS activity and progesterone

Total luteal NOS activity was twofold higher ($P \leq 0.01$) in $\text{PGF}_{2\alpha}$ -treated does than in the control does 6 h after treatment, and maximum activity was reached at 24 h (Fig. 5).

Fig. 3. Western blot analysis probing endothelial nitric oxide synthase (eNOS) protein in corpora lutea of rabbits during $\text{PGF}_{2\alpha}$ -induced luteolysis. Corpora lutea were harvested at 0, 6, 12, 24, and 48 h after injection of $\text{PGF}_{2\alpha}$ analogue, alfaprostol (□), or saline (■) at day 9 of pseudopregnancy. Ten micrograms of protein obtained from corpora lutea of each rabbit were separated in 2% SDS-polyacrylamide gel and transferred on to nitrocellulose membrane. The blots were incubated with monoclonal antibody against human eNOS and developed. (a) Data derived from the densitometric analysis of the eNOS immunoreactive bands. The values (mean $\pm$ SEM), reported in arbitrary units, combine the results from three different rabbits followed by ANOVA with Duncan's test. Bars with different letters are significantly different ($P \leq 0.05$) both within and between groups compared with zero time. (b,c) Two representative immunoblots in which lane MW and eNOS identify molecular mass and endothelial-derived eNOS used as positive standard, respectively. The other lanes identify the corresponding time (h) after (b) saline or (c) $\text{PGF}_{2\alpha}$ injection.

Luteal progesterone content decreased steadily ($P \leq 0.01$) from 302.4 pg mg^{-1} of wet mass at $t = 0$ on day 9 of pseudopregnancy to 58.6 pg mg^{-1} at 48 h after $\text{PGF}_{2\alpha}$ treatment, whereas it remained almost unchanged in control rabbits over the same time period (Fig. 6). The changes in total activity of NOS were inversely correlated with decreases in luteal progesterone content after $\text{PGF}_{2\alpha}$ treatment ($r = -0.88$; $P \leq 0.01$).

Fig. 4. Time-course of inducible nitric oxide synthase (iNOS) protein in corpora lutea of rabbits during prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-induced luteolysis. Corpora lutea were collected at 0, 6, 12, 24 and 48 h after treatment with $PGF_{2\alpha}$ analogue alfaprostol (□), or saline (■) at day 9 of pseudopregnancy. (a) Data derived from the densitometric analysis of the iNOS immunoreactive bands. For each time point, the values (mean $\pm$ SEM), reported in arbitrary units, combine the results from three different rabbits followed by ANOVA with Duncan's test. Bars with different letters are significantly different ($P \leq 0.01$) both within and between groups compared with zero time in luteal iNOS protein expression. (b,c) Typical immunoblots in which lanes MW and iNOS identify molecular mass and mouse macrophage-derived iNOS lysate used as positive standard, respectively. In both panels, the other lanes identify the corresponding time (h) at which corpora lutea were obtained from (b) saline or (c) $PGF_{2\alpha}$.

Similarly, plasma concentrations of progesterone, used as a marker of luteal functionality, decreased gradually in rabbits from 6 h after $PGF_{2\alpha}$ treatment and functional luteolysis was complete 24 h later when plasma progesterone declined to 0.2 ng ml^{-1} ($P \leq 0.01$) (Fig. 7).

Luteolysis in NO-deficient rabbits

At the dose used in the present study, L-NAME did not modify the luteal activity or the lifespan of corpus luteum,

Fig. 5. Total nitric oxide synthase (NOS) activity in rabbit corpora lutea after a bolus injection of $200 \mu\text{g}$ prostaglandin $F_{2\alpha}$ analogue, alfaprostol (□), or saline (■) at zero time on day 9 of pseudopregnancy and at different time intervals. For each time point, the results represent means $\pm$ SEM of three combined values and are referred to mg of wet tissue. Bars with different letters are significantly different ($P \leq 0.01$) both within and between groups compared with zero time. d.p.m.: disintegration per minute.

Fig. 6. Luteal concentrations of progesterone in rabbit corpora lutea after a bolus injection of $200 \mu\text{g}$ prostaglandin $F_{2\alpha}$ analogue, alfaprostol (□), or saline (■) at zero time on day 9 of pseudopregnancy and at different time intervals. For each time point, results represent means $\pm$ SEM of three combined values, and are referred to mg of wet tissue. Bars with different letters are significantly different ($P \leq 0.01$) both within and between groups compared with zero time.

Fig. 7. Plasma concentrations of progesterone in rabbits treated with 1 ml saline (control, ■) and 200 µg prostaglandin $\text{F}_{2\alpha}$ analogue, alprostadol (○) at day 9 of pseudopregnancy. Values are means $\pm$ SEM of three animals per group. Values with different letters are significantly different compared with control and zero time at $P \leq 0.01$.

as the peripheral plasma concentrations of progesterone in NO-deficient rabbits were comparable to those of control rabbits during the days of pseudopregnancy examined (Fig. 8). As expected, mean progesterone concentrations remained unaffected during the following 48 h after saline administration at day 9 of pseudopregnancy. By contrast, the $\text{PGF}_{2\alpha}$ -induced luteolytic response was partially abolished in NO-deficient rabbits as progesterone concentrations failed to decline ($P \leq 0.01$) to basal values within 24–48 h after $\text{PGF}_{2\alpha}$ administration, with a range of 4.5 and 3.2 ng ml⁻¹ (Fig. 8).

Discussion

The main aim of the present study was to verify whether the activity of NOS in corpora lutea is dynamically regulated during luteolysis, which in rabbits normally occurs at about day 15 or day 16 of pseudopregnancy (Browning *et al.*, 1980) but can also be induced by exogenous treatment with $\text{PGF}_{2\alpha}$ (Marcinkiewicz *et al.*, 1992). The present study characterized, at the gene transcript and protein level, the processing of both eNOS and iNOS isoforms during $\text{PGF}_{2\alpha}$ -induced luteolysis. Although the study of the mechanisms of luteolysis has attracted the attention of many investigators (McCracken *et al.*, 2000; Niswender *et al.*, 2000), to the best of our knowledge, this is the first study to investigate the

Fig. 8. Plasma concentrations of progesterone in both nitric oxide (NO)-deficient and control rabbits injected with 2.5 mg prostaglandin $\text{F}_{2\alpha}$ ($\text{PGF}_{2\alpha}$) or saline at day 9 of pseudopregnancy (arrow). NO-deficient rabbits were provided with drinking water containing *N*^ω-nitro-L-argininine methyl ester (L-NAME) (0.6 g l⁻¹) from day 2 of pseudopregnancy onward. Values are means $\pm$ SEM for six animals per group. Asterisks indicate that means of L-NAME-treated rabbits are significantly different from those of control rabbits ($P \leq 0.01$). ■: L-NAME and saline; □: L-NAME and $\text{PGF}_{2\alpha}$; ○: $\text{PGF}_{2\alpha}$.

functional expression patterns of NOS isoforms in luteal tissue during regression of the corpus luteum.

Boiti *et al.* (2002) reported the presence of regulatory mechanisms driving both NOS activity and gene expression during pseudopregnancy, although limited to the formation of the corpus luteum from an early stage to maturation. In the present study, during $\text{PGF}_{2\alpha}$ -induced luteolysis, these proposed mechanisms become much more evident. In fact, eNOS mRNA was shown to decrease 12 h after $\text{PGF}_{2\alpha}$ treatment. The rapid alteration in the expression of eNOS during $\text{PGF}_{2\alpha}$ -induced luteolysis occurs much earlier than the appearance of any abnormality in cellular structure (Koering, 1974) indicating, but not proving, a $\text{PGF}_{2\alpha}$ -induced transcriptional downregulation of the gene or, alternatively, an increased rate of its mRNA degradation. However, the downregulation of the eNOS gene may simply reflect dynamic changes in the luteal cell population after $\text{PGF}_{2\alpha}$ treatment.

A two- to threefold increase in the expression of eNOS protein occurred 12 h after $\text{PGF}_{2\alpha}$ treatment. The decreasing content of luteal eNOS mRNA after treatment with $\text{PGF}_{2\alpha}$ together with the increase of eNOS proteins at comparable times indicates that regulatory mechanisms, as yet unknown, do exist and affect protein synthesis for eNOS at transcription or translation. However, this discrepancy may be due to

gaps in the time-scale used for corpus luteum collection. Several authors have demonstrated that $\text{PGF}_{2\alpha}$ treatment rapidly downregulates the gene expression encoding different steroidogenic key enzymes without affecting their activities (Juengel *et al.*, 1994; Rodgers *et al.*, 1995). eNOS, being constitutive, does not depend upon new protein synthesis to rapidly synthesize and release NO, but increasing experimental evidence indicates that its expression in several types of cell can be dynamically regulated by various factors, such as gonadotrophins, oestrogen, cytokines and prostaglandins (Weiner *et al.*, 1994; Van Voorhis *et al.*, 1995; Jablonka-Shariff and Olson, 1997; Forstermann *et al.*, 1998).

Compared with eNOS, mRNA encoding iNOS was barely detectable and remained unaffected during $\text{PGF}_{2\alpha}$ -induced luteolysis. Moreover, transcripts of iNOS mRNA were expressed without being exogenously induced and were greatly upregulated in the regressing corpus luteum during the first 6 h after $\text{PGF}_{2\alpha}$ administration. Typically, iNOS is regulated mainly at gene expression and requires new protein synthesis to catalyse the formation of NO, which is also produced in larger amounts and for longer periods of time compared with the constitutive isoforms. iNOS is known to be upregulated by several cytokines, such as interleukin 1, interferon γ and tumour necrosis factor α (Forstermann *et al.*, 1995).

Whether the increase in luteal NOS isoforms during induced luteolysis is associated with increases in the expression of eNOS and iNOS mRNAs as a result of transcriptional upregulation in the first few hours after $\text{PGF}_{2\alpha}$ treatment or with greater protein stability still remains to be verified.

The increased expression for iNOS detected within a few hours of $\text{PGF}_{2\alpha}$ treatment may reflect the intense immune cell activity associated with luteal demise and active vascularization. In fact, macrophages have been identified in the corpus luteum of most species as a normal component of the developing corpus luteum (Hehnke *et al.*, 1994; Olson and Townson, 2000; Pate and Keyes, 2001; Townson *et al.*, 2002), and increased numbers of macrophages during luteal regression have been demonstrated in rabbits (Koering, 1974). It should be noted that during spontaneous luteal regression, all morphological studies performed to date on rabbit corpora lutea have described the development of an abundant vascular system with capillaries larger than usual (Koering and Thor, 1978; Dharmarajan *et al.*, 1988). Moreover, late corpora lutea continue to display active DNA synthesis especially in their micro-vascular sept (Nicosia *et al.*, 1995). This finding may also explain the relatively high expression of eNOS protein found in corpora lutea at 48 h after $\text{PGF}_{2\alpha}$ treatment in the present study.

When compared with synchronous untreated control rabbits, total NOS activity increased markedly after $\text{PGF}_{2\alpha}$ administration remaining higher throughout the

subsequent 48 h, whereas luteal progesterone concentrations, used as a marker of functional luteolysis, gradually decreased during the same time period, as did peripheral plasma progesterone concentrations. In rats, iNOS activity was found to be greater than that of the eNOS isoform at all stages of pseudopregnancy and also in regressing corpora lutea (Motta and Gimeno, 1997). Motta *et al.* (2001a) also found that $\text{PGF}_{2\alpha}$ treatment *in vivo* enhanced NOS activity in rat corpus luteum. The present study has not attempted to differentiate the specific contribution to total activity between the two isoforms and, therefore, it is not possible to exclude the fact that the increased production of NO observed during $\text{PGF}_{2\alpha}$ -induced luteolysis is derived from iNOS. Similarly, it is unclear whether translational upregulation of both NOS isoforms found here is sufficient to explain the substantial increase of activity observed in these corpora lutea during $\text{PGF}_{2\alpha}$ -induced luteolysis. In addition to substrate supply, post-translational mechanisms cannot be ruled out in the control of NOS activity in corpora lutea of rabbits.

Although $\text{PGF}_{2\alpha}$ is the principal luteolytic hormone in domestic animals, such as rabbits, it is becoming increasingly apparent that other factors of intra-ovarian origin have the potential to modulate luteal demise. The results of the present study are consistent with the working hypothesis that functional luteolysis in rabbit corpus luteum is evoked by $\text{PGF}_{2\alpha}$ through an upregulation of NOS activity and proteins, mediated via phospholipase C and Ca^{2+} mobilization (Boiti *et al.*, 2001) and induced by other factors, probably cytokines. NO, besides exerting an inhibitory effect on mitochondrial chain P450_{scc} enzymes (Moncada *et al.*, 1991), may also affect other key enzymes, such as cyclooxygenase, promoting prostaglandin production from arachidonic acid, and ADP-ribosylating enzymes (Gow and Ischiropoulos, 2001). After $\text{PGF}_{2\alpha}$ administration, overproduction of NO 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.*, 2001b) and DNA damage (Beckman *et al.*, 1990). Moreover, abnormally high concentrations of NO also play a major role in promoting the intracellular mechanisms of the apoptotic pathways (Kim *et al.*, 1999). However, it must be stressed that a number of details of the mechanisms regulating corpus luteum lifespan are still poorly understood, including the exact sequence of events after receptor binding by $\text{PGF}_{2\alpha}$. Nevertheless, there is good agreement between these *in vivo* and our previous *in vitro* experiments. In fact, in our *in vitro* model, independent of the luteal stages, whenever total NOS-like activity was significantly upregulated by $\text{PGF}_{2\alpha}$, a functional luteolytic response was observed as demonstrated by a marked reduction of progesterone production (Gobbetti *et al.*, 1999; Boiti *et al.*, 2000). In rabbit luteal tissue *in vitro*, NO inhibits progesterone production in a dose-dependent manner (Gobbetti

et al., 1999). In addition, Motta and Gimeno (1997) demonstrated that NO is involved in the regression of corpora lutea isolated from ovaries of pseudopregnant rats during the luteolytic phase. Conversely, in immature rats, NO was found to reverse inhibition of ovarian progesterone secretion induced by PGF_{2α} *in vitro* (Dong *et al.*, 1999).

The L-arginine derivative L-NAME has been used widely to inhibit constitutive NOS in different biological systems both *in vitro* and *in vivo* to indirectly verify the role of NO in reproductive function. It has been shown that intraluteal administration of L-NAME on days 17–18 of the oestrus cycle prolonged the functional life of bovine corpus luteum up to day 25 of the oestrous cycle (Jaroszewski and Hansel, 2000). In female mice lacking the gene for eNOS, the duration of the oestrus cycle was reduced, as was the number of sites of ovarian rupture (Drazen *et al.*, 1999).

In conclusion, these studies have provided the first line of evidence that genes encoding both eNOS and iNOS are regulated in the rabbit corpus luteum during PGF_{2α}-induced luteolysis, thereby further supporting the hypothesis of a functional role of the luteal NO-NOS system *in vivo*. The upregulation of luteal NOS activity observed after PGF₂ treatment and the inhibition of PGF₂-induced luteolysis in NO-deficient rabbits indicate that NO may be a potential mediator of luteal regression in rabbits, although its direct or indirect autocrine and paracrine action remains to be fully elucidated.

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