Nitric oxide synthase acutely regulates progesterone production by *in vitro* cultured rabbit corpora lutea

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Abstract

We examined the presence and the regulation of nitric oxide (NO) synthase (NOS) using *in vitro* cultured corpora lutea (CL) obtained from rabbits at days 4 and 9 of pseudopregnancy. The role of NO and NOS on steroidogenesis was also investigated using the same CL preparations after short-term incubations (30 min and 2 h) with the NO donor, sodium nitroprusside (NP), the NOS inhibitor, Nω-nitro-L-arginine methyl ester (L-NAME) and prostaglandin (PG) F-2α. The basal NOS activity was greater in CL at day 4 than at day 9, and was also differently modulated by PGF-2α, depending on the age of the CL. The addition of PGF-2α to day 4 CL had no effect, but PGF-2α on day 9 caused a threefold increase in NOS activity. NP caused a two- to fivefold decrease in release of progesterone from CL of both ages, and this inhibitory effect on steroidogenesis was reversed by L-NAME. All treatments failed to modify basal androgens and 17β-oestradiol was not detectable in either control or treated CL. These results suggest that NO is effectively involved in the regulation process of steroidogenesis, independently of 17β-oestradiol. PGF-2α had no effect on day 4, but induced luteolysis on day 9, by reducing progesterone ($P \leq 0{\cdot}01$) to about 18% of control. The luteolytic action of PGF-2α was completely reversed by co-incubation with L-NAME, thus supporting the hypothesis that luteolysis is mediated by NO. The addition of NP or L-NAME did not modify the *in vitro* release of PGF-2α. We hypothesised that PGF-2α upregulates NOS activity and, consequently, the production of NO, which acutely inhibits progesterone release from day 9 CL of pseudopregnant rabbits.

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Introduction

Over the past 10 years, nitric oxide (NO) has emerged as a new intra- and intercellular signalling molecule regulating a large array of biological actions, including vasodilatation, neurotransmission and cytotoxicity, under both physiological and pathophysiological conditions (Moncada *et al.* 1991, Snyder & Bredt 1992, Schmidt & Walter 1994).

In vivo, NO is generated by the oxidation of L-arginine in a multistep reaction catalysed by NO synthases (NOS), a family of isoenzymes that require NADPH, calcium, and calmodulin to yield NO and L-citrulline (Hibbs *et al.* 1987, Bredt & Snyder 1990). According to their expression in cells, the NOS isoforms have been divided into constitutive and inducible NOS, referred to as cNOS and iNOS, respectively. cNOS is expressed fairly constantly, whereas iNOS is not usually present in cells until they are exposed to cytokines (interferon-γ, interleukin-1β) or other immunostimulants of bacterial origin (Stuehr & Marletta 1985). The binding of calmodulin to the enzyme is essential for the activity of all NOS isoforms in a particular process, which could, however, be either Ca^{2+}-dependent or Ca^{2+}-independent. cNOS requires Ca^{2+} for the reversible binding of calmodulin to the enzyme to become active and generate NO in a carefully regulated process initiated by receptors or physical stimulation. Conversely, iNOS, once expressed, remains in an activated state, regardless of whether intracellular calcium is tightly bound to calmodulin as a prosthetic group (Nathan & Xie 1994).

NOS activities were first reported in cytokine activated macrophages (Stuehr & Marletta 1985), and soon thereafter in endothelium (Ignarro *et al.* 1987, Palmer *et al.* 1988) and neurones from cerebellum (Garthwaite *et al.* 1988), where NO has been shown to regulate antimicrobial and antitumoral activities, vasodilatation and neurotransmission. Subsequently, NOS was also identified in other tissue cells such as smooth muscle and hepatocyte (Busse & Mulscha 1990, Nakayama *et al.* 1992).

More recently, NOS has been found both in ovarian stroma and follicular granulosa cells of human, rat and rabbit ovary, where it has been shown to regulate steroidogenesis (Van Voorhis *et al.* 1994, Chatterjee *et al.* 1996, Hesla *et al.* 1997, Jablonka-Shariff & Olson 1997). NO has been shown to inhibit steroidogenesis in the rat

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testes (Adams *et al.* 1992) and cultured Leydig cells of rat (Welch *et al.* 1995) and mouse (Pomerantz & Pitelka 1998). In both rabbits and rats, the ovarian NO/NOS system has also been shown to be involved in the process and rate of ovulation (Shukovski & Tsafriri 1995, Bonello *et al.* 1996, Zackrisson *et al.* 1996, Hesla *et al.* 1997, Yamauchi *et al.* 1997). All these findings, therefore, suggest that NO may have important implications in the physiology of the ovary by controlling several functions such as steroidogenesis, ovulation and luteolysis.

In the present study, we examined the potential role of NO as a luteolytic effector by monitoring progesterone output during short-term incubation of *in vitro* cultured corpora lutea (CL) obtained from rabbits on day 4 and day 9 of pseudopregnancy. These two sets of CL, having different *in vivo* responsiveness to prostaglandin (PG) F-2α (Carlson & Gole 1978, Kehl & Carlson 1981) or to its analogue, alfaprostol (Boiti *et al.* 1998), were selected as a model for investigating the cellular mechanism involved in the luteolysis process. The effect of NO was assessed by short-term incubations of CL with a specific NO donor, sodium nitroprusside (NP), and an inhibitor of NOS, Nω-nitro-L-arginine methyl ester (L-NAME). Some of the characteristics of the response by *in vitro* cultured rabbit CL to NO were investigated using PGF-2α as reference compound, given its well-known luteolytic action.

Materials and Methods

Animal and hormonal regimen

For the experiment, 12 mature female New Zealand White rabbits, weighing 3–4 kg, were housed individually in an indoor facility under controlled conditions of light (14 h light : 10 h darkness) and temperature 18 °C. Each animal had free access to food and water. Pseudopregnancy was induced with 20 IU pregnant mare's serum gonadotrophin (i.m.) followed 3 days later by an i.m. injection of 0·8 μg gonadotropin-releasing hormone (GnRH) analogue (Receptal, Roussel–Hoechst, Munich, Germany). Previous experiments in our laboratory showed that this hormonal protocol was effective in inducing ovulation in rabbit does (Stradaioli *et al.* 1997). The day of GnRH injection was designated day 0. Rabbits were killed on day 4 and on day 9 of pseudopregnancy by cervical dislocation. CL were promptly removed from the ovary and the non-luteal tissue was dissected away from each CL with fine forceps under stereoscopic magnification. All 4-day-old CL were pooled together for each experiment, as were those collected from day 9 of pseudopregnancy.

In vitro *incubations*

CL were immediately quartered and randomly distributed (one CL/well) into incubation wells (Becton Dickinson Co., Clifton, NJ, USA) in 1 ml culture medium 199 with Earle's Balanced Salt Solution (Gibco, Grand Island, NY, USA) containing 2·2 mg/ml sodium bicarbonate, 2·3 mg HEPES (Sigma, St Louis, MO, USA), and 1% BSA (Sigma), referred to here as M199. Each set of incubation wells was divided into seven experimental groups of two wells as follows: (I) M199 alone as control; (II) M199 plus PGF-2α (3 μM); (III) M199 plus NP (3·5 μM); (IV) M199 plus L-NAME (1 μg); (V) M199 plus PGF-2α (3 μM) plus L-NAME (1 μg); (VI) M199 plus PG synthase inhibitor (acetylsalicylic acid, ASA) (1 μg); (VII) M199 plus NP (3·5 μM) plus ASA (1 μg). NP used as a specific NO donor and L-NAME, as NOS inhibitor, were both purchased from Sigma as PGF-2α and ASA. Culture plates were incubated at 37 °C. The media of the two wells for each treatment were collected after 30 min and 2 h of incubation and stored immediately at −20 °C for later determination of progesterone, androgens, 17β-oestradiol, and PGF-2α. CL were weighed and stored immediately at −20 °C for later determination of NOS activity. The experiment was repeated with 4- and 9-day-old CL. Tests on three parallel incubation sets were carried out. We chose the incubation conditions and the minimum effective doses of the substances used in the present *in vitro* study on the basis of preliminary evidence (Fig. 1).

Determination of NOS activity

NOS activity was determined in the CL used for *in vitro* incubations by monitoring the conversion of [^{3}H]L-arginine into [^{3}H]L-citrulline, with a modified method described previously (Bush *et al.* 1992, Burnett *et al.* 1995). Half the CL of each well were homogenized in 1 ml cold fresh homogenising buffer (50 mM Tris, 1 mM EDTA and 1 mM EGTA, pH 7·4), and centrifuged at 20 000 ***g*** for 60 min at 4 °C. Twenty-five microlitres supernatant and 100 μl incubation buffer (1·5 mM NADPH, 1 mM $CaCl_2$) containing 150 000 d.p.m. [2,3-^{3}H]L-arginine (Sigma, sp. act. 30–40 Ci/mmol) were added to the incubation tube. After 30 min incubation at room temperature, the enzymatic reaction was stopped by the addition of 2 ml blocking buffer (20 mM HEPES, 2 mM EDTA, pH 5·5). The mixture was applied to a pre-equilibrated column (20 mM sodium acetate, 2 mM EDTA, 0·2 mM EGTA, pH 5·5; 1 cm diameter) containing 1 ml Dowex AG50W-X8 (Sigma), and the material was eluted with 2 ml water. [^{3}H]L-Citrulline was quantified in a liquid scintillation system LS 1801 (Beckman Instr., Fullerton, CA, USA). Additional determinations were performed in the presence of excess L-NAME to verify the specificity of the assay for production of [^{3}H]L-citrulline by NOS (data not shown). Protein concentration was determined by Bio-Rad Protein Assay Kit (Bio-Rad Lab., Hercules, CA, USA).

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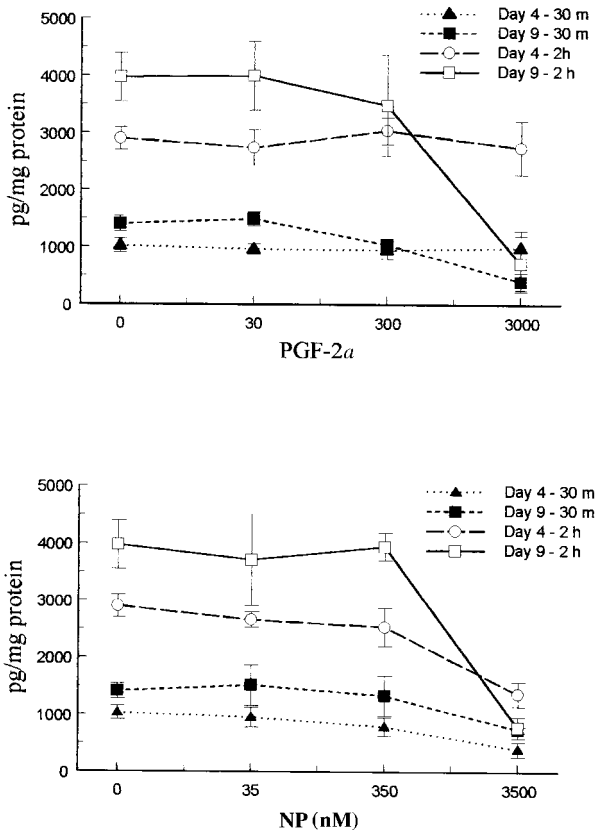

Figure 1 *In vitro* effects of different concentrations of PGF-2α and an NO donor (NP) on progesterone release by rabbit CL collected on days 4 and 9 of pseudopregnancy and incubated for 30 min or 2 h. Values are means ± S.D. of three determinations. The effects produced by PGF-2α at a concentration of 3000 nM (upper panel) were significantly different ($P \leq 0{\cdot}01$) from basal values after 30 min and 2 h of incubation only on day 9 CL (one-way ANOVA). NP at a concentration of 3500 nM significantly ($P \leq 0{\cdot}01$) reduced progesterone release in both day 4 and day 9 CL.

PGF-2α and steroid radioimmunoassay

PGF-2α, progesterone, androgens and 17β-oestradiol were determined in the medium samples using the RIA previously reported (Gobbetti *et al.* 1992). Intra- and interassay coefficients of variation and minimum detectable doses were: PGF-2α, 7%, 13%, 17 pg; progesterone, 5%, 9%, 10 pg; androgens, 5%, 12%, 9 pg; 17β-oestradiol, 9%, 11%, 25 pg. PGF-2α and testosterone antisera were provided by Dr G F Bolelli (CNR-Institute of Normal and Pathologic Cytomorphology, University of Bologna, Italy) and Dr F Franceschetti (CNR-Physiopathology of Reproduction Service, University of Bologna, Italy). Progesterone and 17β-oestradiol antisera were purchased from Sigma. Testosterone was not separated from 5α-dihydrotestosterone and, as the antiserum used is not specific, the data are expressed as androgens. Tritiated PGF-2α, progesterone, and testosterone were purchased from Amersham Int. (Amersham, Bucks, UK), and non-radioactive PGF-2α, progesterone, and testosterone from Sigma.

Statistics

Data relative to release of hormones and enzymatic activities were analysed by ANOVA (Sokal & Rohlf 1981), followed by Duncan's multiple range test (Duncan 1955).

Results

The response curves for the agonists used, PGF-2α and NP, are shown in Fig. 1. The NO donor-induced inhibition of progesterone release by isolated CL of day 4 and day 9 was both time- and concentration-dependent; the dose-dependent effect obtained by increasing concentrations of PGF-2α was observed only in day 9 CL.

NOS activity in in vitro *cultured CL*

The mean ± S.D. basal NOS activities in the CL incubated with medium alone for 2 h were 17 300 ± 2340 and 11 250 ± 1260 d.p.m./mg wet tissue at days 4 and 9 respectively. To normalise the data obtained from different preparations, results were expressed as a mean percentage of the basal NOS activity (Fig. 2).

On day 4 of pseudopregnancy, the addition of PGF-2α (3 μM) to CL preparations had no effect on NOS activity after 2 h incubation, but on day 9 CL it induced an increase to more than 250% of baseline activity ($P \leq 0{\cdot}01$) after the same time interval (Fig. 2).

Treatment with ASA (1 μg) almost doubled ($P \leq 0{\cdot}01$) basal NOS activity after 2 h incubation in day 4 CL only. Its co-incubation with NP resulted in a similar response. In contrast, in day 9 CL, NOS activity was drastically reduced to about 18% of its basal value by incubation with both ASA and ASA plus NP (Fig. 2).

The addition of L-NAME (1 μg) resulted in an almost 90–95% inhibition of NOS activity in all the treatments tested, independently of the age of the CL (data not shown).

Similar results were observed in CL preparations after 30 min incubation on both day 4 and day 9 of pseudopregnancy (data not shown).

Effect of NO donor and PGF-2α on steroid release by in vitro *cultured CL*

The mean ± S.D. basal release of progesterone produced by CL incubated with medium alone for 2 h were 2·9 ± 0·2 and 3·9 ± 0·4 ng/mg wet tissue at 4 and 9 days

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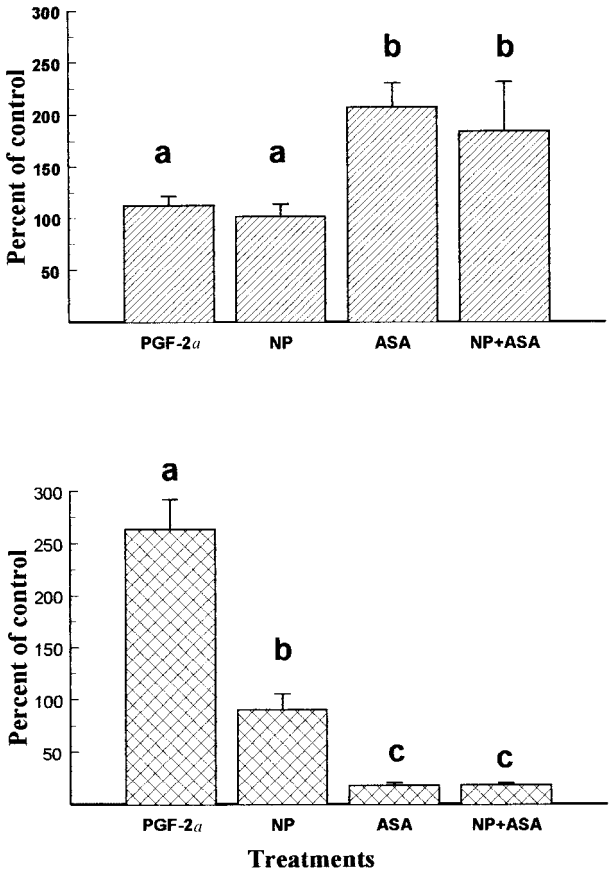

Figure 2 *In vitro* effects of PGF-2α, an NO donor (NP), a PG synthase inhibitor (ASA), and NP plus ASA on NOS activity by rabbit CL collected on days 4 (upper panel) and 9 (lower panel) of pseudopregnancy and incubated for 2 h. Results are the mean ± S.D. of three values and are expressed as a percentage of the control values: means ± S.D. of NOS activities in the CL incubated with medium alone were 17 300 ± 2340 and 11 250 ± 1260 d.p.m./mg wet tissue at days 4 and 9 respectively. Within each panel, different letters above bars indicate significantly different values ($P \leq 0{\cdot}01$).

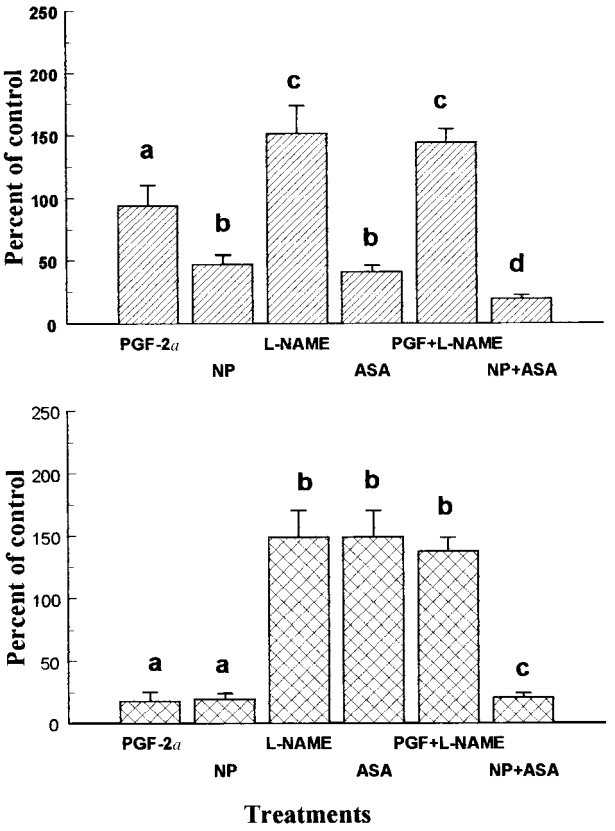

Figure 3 *In vitro* effects of PGF-2α, an NO donor (NP), an NOS inhibitor (L-NAME), a PG synthase inhibitor (ASA), PGF-2α plus L-NAME, and NP plus ASA, on release of progesterone by rabbit CL collected on days 4 (upper panel) and 9 (lower panel) of pseudopregnancy and incubated for 2 h. Results are the mean ± S.D. of three values and are expressed as a percentage of the control value. Progesterone concentrations (mean ± S.D.) in control wells were 2·9 ± 0·2 and 3·9 ± 0·4 ng/mg wet tissue in day 4 and day 9 CL respectively. Within each panel, different letters above bars indicate significantly different values ($P \leq 0{\cdot}01$).

respectively. To normalise the data obtained by different preparations, results were expressed as a mean percentage of basal release of progesterone (Fig. 3).

The addition of NP to isolated CL of 4 and 9 days of pseudopregnancy resulted in a decrease in the release of progesterone; this inhibitory effect was greater on CL of day 9 than on those of day 4 (Fig. 3).

In CL preparations of day 4, the addition of PGF-2α did not affect progesterone production, which remained unchanged with respect to control after 2 h incubation. CL of day 9 of pseudopregnancy showed a completely different *in vitro* acute response to PGF-2α, which exhibited a clear luteolytic effect by reducing ($P \leq 0{\cdot}01$) the progesterone release to about 18% of control. This effect of PGF-2α on CL of day 9 was not only completely prevented by treatment with ASA, but also reversed. In fact, when endogenous PG synthesis was abolished by treatment with ASA, progesterone production by day 9 CL increased ($P \leq 0{\cdot}01$) by almost 50% over that of control. The addition of ASA to NP potentiated the luteolytic effect of the NP itself, as assessed by progesterone concentrations, only in CL of day 4 (Fig. 3).

Similar results were observed in CL preparations after 30 min incubation on both day 4 and day 9 of pseudopregnancy (data not shown).

Treatments of luteal tissue for short or longer periods of time with the entire concentration range of the substances tested here failed to modify basal concentrations of androgens secreted in the medium (data not shown); they remained very close to that found in non-stimulated control CL, independently of the increasing age of the CL. In our *in vitro* system, 17β-oestradiol concentrations were not detectable in either control or treated wells.

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Effect of NOS inhibitor and PGF-2α on steroid release by in vitro *cultured CL*

The *in vitro* influence of NO on steroidogenesis was also examined on CL of the same age by means of L-NAME and L-NAME co-incubated with PGF-2α. The exogenous administration of the specific L-NAME (1 μg) caused a significant 50% increase ($P \leq 0{\cdot}01$) in control release of progesterone at 2 h on both day 4 and day 9 CL (Fig. 3). Similarly, PGF-2α added to L-NAME did not alter progesterone secretions by day 4 and day 9 CL during 2 h of static incubation, which were 37–44% greater than those in controls (Fig. 3). Also in these cases, total androgens were not affected by any treatment, and 17β-oestradiol concentrations were not measurable. Similar results were observed in CL preparations after 30 min incubation on both day 4 and day 9 of pseudopregnancy (data not shown).

PGF-2α releases by in vitro *cultured CL*

In non-stimulated *in vitro* cultured luteal tissue, basal release of PGF-2α after 2 h incubation was greater ($P \leq 0{\cdot}01$) in CL collected at day 9 of pseudopregnancy (869 ± 146 pg/mg wet tissue) than at day 4 (329 ± 44 pg/mg wet tissue). The addition of NP or L-NAME did not modify the *in vitro* release of PGF-2α (Fig. 4), whereas ASA decreased it ($P \leq 0{\cdot}01$).

Similar results were observed in CL preparations after 30 min incubation on both day 4 and day 9 of pseudopregnancy (data not shown).

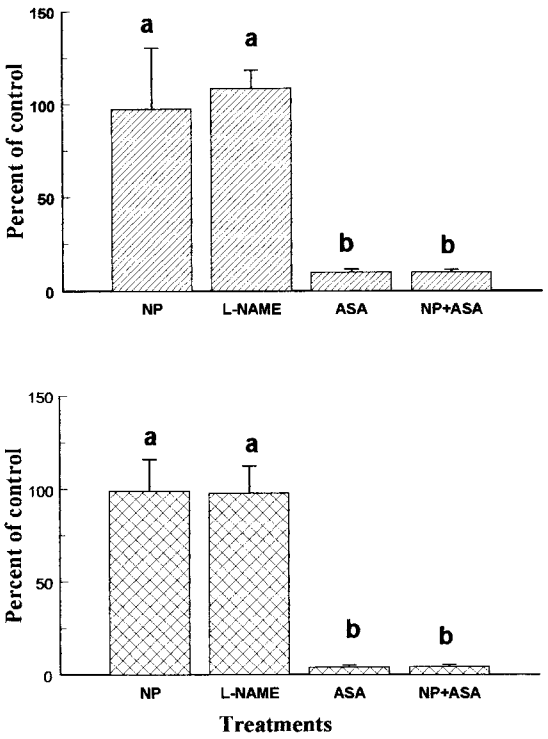

Figure 4 *In vitro* effects of an NO donor (NP), an NOS inhibitor (L-NAME), a PG synthase inhibitor (ASA), and NP plus ASA on PGF-2α release by rabbit CL collected on days 4 (upper panel) and 9 (lower panel) of pseudopregnancy and incubated for 2 h. Results are the mean ± S.D. of three values, and are expressed as a percentage of the control value. The mean ± S.D. PGF-2α concentrations in control wells were 329 ± 44 and 869 ± 146 pg/mg wet tissue at days 4 and 9 respectively. Within each panel, different letters above bars indicate significantly different values ($P \leq 0{\cdot}01$).

Discussion

To the best of our knowledge, we have demonstrated for the first time the presence of NOS activity in the CL of pseudopregnant rabbit, where it seems to exert a regulatory role on steroidogenesis. In our experimental model, the NOS total activity decreased with increasing age of the CL: those collected in the early-luteal phase of pseudopregnancy, at day 4, had greater basal contents of NOS enzymes than did CL collected in the mid-luteal phase, at day 9. Although the cellular source of the NO produced was not assessed here, this decremental NOS activity could be explained by the continuous remodelling process between large and small luteal cell populations that also characterises CL development in the rabbit (Hoyer *et al.* 1986), or by local hormonal action. This could, however, be better investigated by the immunocytochemical cellular localisation of NOS enzymes, using antisera isoforms specific for both endothelial and inducible isoforms, both in cultured cell populations isolated from CL of different ages and in sections of the CL itself.

Expression of endothelial NOS has also been demonstrated by Van Voorhis *et al.* (1994) in human ovarian follicular cells by extraction of endothelial NOS (eNOS)-specific mRNA and by immunocytochemistry. Both isoforms of NOS were identified by Olson *et al.* (1996) in ovaries obtained from hypophysectomized, hormone-treated rats. According to these authors, the proportion of cells expressing eNOS, mainly steroidogenic and endothelial cells, was probably much greater than that of cells, such as macrophages, showing a positive staining for iNOS.

Interestingly, from our data obtained by *in vitro* studies, it emerged that PGF-2α modulates the luteal NOS activity differently depending on the age of CL: treatment of day 4 CL with PGF-2α had no effect on NOS activity, whereas, in similarly cultured CL collected at day 9 of pseudopregnancy, PGF-2α caused a marked increase in NOS activity. This effect evoked by PGF-2α on the *in vitro* cultured CL tissue preparations was also confirmed by the administration of ASA.

An increasing amount of experimental work supports the hypothesis that NO may also have a significant role in the regulation of steroidogenesis, not only in the ovary, but

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also in the testis (Adams *et al.* 1992), Leydig cell (Welch *et al.* 1995, Pomerantz & Pitelka 1998) and adrenal axis (Palacios *et al.* 1989). By studying the effect of NO on *in vitro* cultured granulosa luteal cells obtained from women undergoing *in vitro* fertilisation procedures, Van Voorhis *et al.* (1994) found that this compound reduced the synthesis of both 17β-oestradiol and progesterone. Comparable results were also obtained by Olson *et al.* (1996) by using *in vitro* cultured ovarian cells obtained from hypophysectomized, hormone-treated rats. They observed that the basal production of NO was much greater in cells obtained from luteinized ovaries than that in cells at all other ovarian developmental stages. Specific inhibition of endogenous NO synthesis by means of aminoguanidine in the 0–1000 μM range resulted in a significant two- to threefold increase in the concentration of 17β-oestradiol, whereas production of progesterone remained unaffected. In contrast, NP (0–400 μM) reduced 17β-oestradiol to about 25% of the control, but progesterone concentrations were reduced to a much lesser extent. On this basis, these authors supposed that NO of ovarian origin might control the life-span of the CL by regulating the 17β-oestradiol concentration that is required to maintain adequate progesterone synthesis and normal luteal function.

In our experiments, NP acutely decreased progesterone secretion by *in vitro* cultured CL collected from pseudopregnant rabbits in a time-dependent manner, independently of their developmental stage. Furthermore, this inhibitory effect on steroidogenesis was reversed by treatment with L-NAME, as progesterone output increased. It remains to be established, however, if this increased progesterone release is due to an actual synthesis rather than to leakage from dying luteal cells as suggested by Dharmarajan *et al.* (1994) and Guo *et al.* (1998), who reported that rabbit CL undergo spontaneous apoptosis within a few hours when cultured in serum-free medium. Also, an anti-apoptotic effect of the NOS inhibitor, L-NAME, on the *in vitro* cultured CL could not be excluded, as it has recently been established that NO has an active role in transducing apoptotic pathways that lead to cell death (Brune *et al.* 1998*b*). These results provide the initial evidence that NO is also effectively involved in the regulation process of steroidogenesis in rabbit luteal tissue, independently of age. Contrary to rat and human culture systems, however, this regulatory process is independent of 17β-oestradiol. In fact, we did not find any effect of NO on either total androgens or 17β-oestradiol production, possibly because, in our experimental model, the incubation time was not long enough for the accumulation of measurable amounts of 17β-oestradiol in the medium.

Several hormones are known to regulate, both *in vivo* and *in vitro*, the synthesis of progesterone by CL. In rabbits, 17β-oestradiol has been identified as the principal luteotrophic hormone, as CL are totally dependent upon it. Until recently, it was believed that oestradiol was produced by the ovarian follicles, as rabbit CL had no aromatase activity. Only recently have Arioua *et al.* (1997) shown that cultured rabbit luteal tissue from hyperstimulated pseudopregnant animals exhibits an intrinsic aromatase activity, producing oestradiol. However, the question as to whether 17β-oestradiol may contribute to the regulation of progesterone synthesis still remains unanswered.

Administration of PGF-2α to *in vitro* cultured CL collected from day 9 of pseudopregnancy resulted in a clear-cut decrease in progesterone production after 2 h incubation, which was, however, already evident after 30 min incubation. In contrast, McLean *et al.* (1987) did not show any altered secretion of progesterone in response to the addition of PGF-2α to rabbit luteal cells obtained at day 10 of pseudopregnancy. Similarly, Dharmarajan *et al.* (1989) found that PGF-2α did not affect progesterone in the *in vitro* perfused rabbit ovary at day 11 or day 18 of pseudopregnancy. Contradictory results have sometimes been reported concerning control of luteal function when applied to *in vitro* studies. These discrepancies are mainly related to the difference in the *in vitro* technique used. Several studies indicate that rabbit luteal cells maintain their viability and can be successfully cultured for several hours or days after dissociation and dispersion using collagenase. However, enzymatic dissociation and culture of luteal cells might alter the properties of these cells with respect to the responsiveness to luteotropic or luteolytic hormones (McLean & Miller 1985). Therefore, the specific *in vitro* technique used may explain the several reported discrepancies in those experiments carried out with *in toto* or minced CL, and similarly challenged with the same hormones in short-term incubation. In our experiment, the inhibitory effect induced by PGF-2α was completely reversed by co-incubation with L-NAME. A similar antisteroidogenic effect on progesterone secretion was obtained by treatment with NP. The simultaneous administration of ASA did not influence the demise of progesterone production induced by NP. In contrast, PGF-2α had no effect on day 4 CL. Taken together, all these data strongly support the hypothesis that the *in vitro* luteolytic effect triggered by exogenous PGF-2α is mediated by NO. At present, no information is available concerning the signalling mechanism activating NO production induced by luteolytic factors after receptor binding, or regulating the expression of NOS during the CL development of pseudopregnant rabbit. NO has been found to exert a stimulatory activity for several intracellular signalling pathways (cGMP), but also an inhibitory effect on mitochondrial chain P450 enzymes or, more generally on mitochondrial function (Moncada *et al.* 1991). However, the known ability of PGF-2α to increase intracellular Ca^{2+} could explain the activation of NOS to release NO. In fact, constitutive NOS requires Ca^{2+} for the reversible binding of calmodulin to the enzyme to become active and generate NO in a carefully regulated process initiated

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by receptors or physical stimulation. This working hypothesis is also supported by the finding that PGF-2α increases total NOS activity in day 9 CL. Therefore, although the acute inhibition of progesterone synthesis induced by PGF-2α could be attributable to activation of the protein kinase C pathway, inactivation of cAMP in the small luteal cells, or both (Wiltbank *et al.* 1991), the involvement of NO cannot be ruled out.

Endogenous or exogenous NO may also have many other intracellular targets such as ADP-ribosylating enzymes, the haem-containing enzyme, cyclo-oxygenase, promoting PG production from arachidonic acid, and the mitochondrial cytochrome P450 steroidogenic enzymes (cholesterol side-chain cleavage). NO derived from eNOS has been shown to bind to the haem prosthetic group of soluble guanylate cyclase and increase intracellular cGMP concentrations, which have been associated with vascular smooth muscle relaxation. This concomitant increase in cellular cGMP has also been verified in cultured human granulosa dispersed cells (Van Voorhis *et al.* 1994) treated with NP and in many other cell culture systems in which NOS is normally expressed. However, the role of cGMP as a second messenger in the regulation of steroidogenesis remains unclear. In fact, according to Van Voorhis *et al.* (1994), the inhibitory effect induced by NO on 17β-oestradiol secretion is independent of the increase in cGMP, as addition of cGMP analogues does not inhibit steroid synthesis. Progesterone synthesis seems to be controlled, at least in part, by the NOS/NO system. The major cellular signal transduction mechanisms of NO involved in several biological actions are mediated by its binding to iron-containing enzymes. Although the precise biochemical nature of the inhibition of steroidogenesis by NO still remains unknown, some hypotheses can be made on the basis of available knowledge. NO may act by selectively inhibiting the cytochrome P450SCC enzyme, which controls cholesterol side-chain cleavage in the inner mitochondrial membrane. Also, 17β-oestradiol synthesis appears to be regulated by NO, which exerts its anti-steroidogenic action by inhibiting the cytochrome P450 aromatase enzymes in the granulosa luteal cells. In the rabbit, however, our data failed to confirm this.

It is now well established that long-lasting NO generation leads to apoptotic cell death. However, accumulating evidence suggests that the transducing pathways activated by NO are directed not only to cytotoxicity, but also to cell protection, by up-regulation of protective proteins and controlled NO/O_2^- (superoxide) interaction, together with several other biochemical mechanisms (Brune *et al.* 1998*a*). Therefore, the possible anti-apoptotic effects of NO on *in vitro* cultured CL, triggered by the addition of the NO donor, NP, or blocked by the NOS inhibitor, L-NAME, should also be investigated.

Various reports (Hansel & Dowd 1986, Schlegel *et al.* 1988) have documented that the CL of rabbit also produce relatively large amounts of several PGs, including PGF-2α and PGE-2, raising questions about their role. In our study, we found that the basal amount of PGF-2α released by 9-day-old CL was almost twice that of CL obtained at day 4 of pseudopregnancy. NO has been found to activate cyclo-oxygenase enzymes (Salvemini *et al.* 1993) that control PG production from arachidonic acid, and this mechanism has been associated with ovulation in rabbit (Hesla *et al.* 1997). In our experimental model, however, we were unable to observe any increase in PGF-2α synthesis by cultured CL 2 h after the addition of either NP or L-NAME at any of the dosages tested.

In rabbit, as in most other species, PGF-2α or its analogues evoke a luteolytic effect, inducing a rapid decrease in progesterone production, which is, however, closely related to the age of CL (Marcinkiewicz *et al.* 1992, Boiti *et al.* 1998). Several different hypotheses have been proposed to explain the decreased synthesis of progesterone by CL and the luteal regression induced by PGF-2α: reduced luteal blood flow, downregulation of luteinizing hormone (LH) receptors, uncoupling of the LH receptor from adenylate cyclase, activation of protein kinase C, Ca^{2+} influx, and cytotoxic effect. However, the intra- and intercellular mechanisms by which PGF-2α exerts its luteolytic action are not yet well understood. Difficulties in identifying these mechanisms of action arise from significant differences among species and also from the discrepancies between *in vivo* and *in vitro* studies.

Despite the fact that previous studies have shown that progesterone secretion peaks around day 11 of pseudopregnancy (Dharmarajan *et al.* 1988), for this experiment, as a model for investigating NO in the *in vitro* steroidogenesis, we purposely selected CL from rabbits at 4 and 9 days of pseudopregancy, as the former are totally refractory, and the latter completely responsive *in vivo* to PGF-2α (Carlson & Gole 1978, Kehl & Carlson 1981) or to its analogue, alfaprostol (Boiti *et al.* 1998). In fact, during pseudopregnancy, CL undergoes a continuous remodelling process between large granulosa-derived and small theca-derived steroidogenic luteal cell populations during its development in rabbit (Hoyer *et al.* 1986), which confers on it a differential sensitivity over time to the actions of several luteotrophic and luteolytic hormones. It is now generally accepted that steroidogenesis is controlled by several mechanisms in the different luteal cell types, and it is likely that messengers of any one cell type may influence the synthesis in any other cell type in a paracrine fashion. In the ewe, in which a similar proportion of large and small cell types were found, Fitz *et al.* (1982) demonstrated that the small cells contain the majority of LH receptors, whereas the large ones have the majority of the PGF-2α receptors. Indeed, it is now well established that the stage of CL development dictates the molecular mechanism involved in the process of luteolysis.

In the present study, we did not attempt to differentiate NOS isoforms. The question as to whether NO produced by the rabbit CL under *in vitro* conditions would also apply

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to *in vivo* systems remains unanswered and requires further investigation. Also, the possible mechanisms of NO-induced inhibition of steroidogenesis by *in vitro* cultured rabbit luteal tissue remain to be elucidated. Studies are under way to determine the role of NO in each type of luteal cell obtained both from early- and mid-luteal phase of pseudopregnancy.

In conclusion, from the present data it emerges that NOS activity is differently modulated by PGF-2α in day 4 and day 9 CL. In day 4 CL, PGF-2α does not affect either the activity of this enzyme or progesterone release. In contrast, in day 9 CL, PGF-2α activates NOS with a consequent decrease in progesterone. Taken together, these results suggest a new intracellular mechanism used by PGF-2α in exerting its luteolytic action and regulating the life-span of rabbit CL.

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