Nitric oxide synthase acutely regulates progesterone production by \textit{in vitro} cultured rabbit corpora lutea

A Gobbetti, C Boiti$^1$, C Canali$^1$ and M Zerani

Dipartimento di Biologia Molecolare, Cellulare e Animale, Università di Camerino, Camerino, Italy
$^1$Istituto di Fisiologia Veterinaria, Università di Perugia, Perugia, Italy

(Requests for offprints should be addressed to C Boiti, Istituto di Fisiologia Veterinaria, Università di Perugia, via S Costanzo 4, 06100 Perugia, Italy)

Abstract

We examined the presence and the regulation of nitric oxide (NO) synthase (NOS) using \textit{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-ω-arginine methyl ester (ω-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 1-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 ($P \leq 0.01$) to about 18% of control. The luteolytic action of PGF-2α was completely reversed by co-incubation with ω-NAME, thus supporting the hypothesis that luteolysis is mediated by NO. The addition of NP or ω-NAME did not modify the \textit{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.


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 \textit{et al.} 1991, Snyder & Bredt 1992, Schmidt & Walter 1994).

\textit{In vivo}, NO is generated by the oxidation of ω-arginine in a multistep reaction catalysed by NO synthases (NOS), a family of isoenzymes that require NADPH, calcium, and calmodulin to yield NO and ω-citrulline (Hibbs \textit{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 \textit{et al.} 1987, Palmer \textit{et al.} 1988) and neurones from cerebellum (Garthwaite \textit{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 & Mulsch 1990, Nakayama \textit{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 \textit{et al.} 1994, Chatterjee \textit{et al.} 1996, Hesla \textit{et al.} 1997, Jablonka-Shariff & Olson 1997). NO has been shown to inhibit steroidogenesis in the rat.
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, Zackrission 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 (Stradaioi 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 [3H]-l-arginine into [3H]-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 CaCl₂) containing 150 000 d.p.m. [2,3-3H]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. [3H]-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 [3H]-l-citrulline by NOS (data not shown). Protein concentration was determined by Bio-Rad Protein Assay Kit (Bio–Rad Lab., Hercules, CA, USA).
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 Boletti (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 < 0·01) after the same time interval (Fig. 2).

Treatment with ASA (1 µg) almost doubled (P < 0·01) 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).

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 ≤ 0·01) 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 ≤ 0·01) reduced progesterone release in both day 4 and day 9 CL.
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 \((P \leq 0.01)\) 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 \((P \leq 0.01)\) 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 CL respectively. Within each panel, different letters above bars indicate significantly different values \((P \leq 0.01)\).

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≤0·01) 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≤0·01) 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≤0·01).

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

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 immunocytotoxic chemical 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
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 (Bruno et al. 1998b). 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 Ca2+ could explain the activation of NOS to release NO. In fact, constitutive NOS requires Ca2+ 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. This working hypoth-
esis 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-oxigenase,
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
acellular 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 genera-
tion 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 (Brun et al.
1998a). 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,
t-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 t-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 progeste-
one 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 pseudo-
pregnancy (Dharmarajan et al. 1988), for this experiment,
as a model for investigating NO in the in vitro steroido-
genesis, we purposely selected CL from rabbits at 4 and 9
days of pseudopregnancy, as the former are totally refrac-
tory, and the latter completely responsive in vivo to
PGF-2α (Carlson & Gole 1978, Kehl & Carlson 1981) or
to its analogue, alfa-prostol (Boiti et al. 1998). In fact,
during pseudopregnancy, CL undergoes a continuous
remodelling process between large granulosa-derived and
small theca-derived steroidogenetic 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 con-
trolled 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 propor-
tion 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 vitro 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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