Nitric oxide synthase activity and progesterone release by isolated corpora lutea of rabbits in the early and mid-luteal phases of pseudopregnancy are modulated differently by prostaglandin E-2 and prostaglandin F-2α via adenylate cyclase and phospholipase C

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Abstract

By examining *in vitro* the effects of prostaglandin E-2 (PGE-2) and prostaglandin F-2α (PGF-2α) induced in the corpora lutea (CL) of pseudopregnant rabbits, we have demonstrated that these prostaglandins modulate luteal nitric oxide synthase (NOS) activity and progesterone production differently, depending on the age of the CL. On CL obtained on day 4 of pseudopregnancy (day-4), PGE-2 was found to depress NOS total activity to 13% of control and to significantly increase basal progesterone secretion by 61%, while PGF-2α had no effect. On day-9 CL, PGE-2 was ineffective, but PGF-2α caused a 2·5-fold increase of NOS activity and a marked decrease in progesterone production. Using specific inhibitors, we found that the regulatory actions of PGE-2 *in vitro* are mediated via the adenyl cyclase/protein kinase A (PKA) second messenger system, while the PGF-2α-induced luteolytic effects on day-9 CL depend upon activation of the phospholipase C/protein kinase C (PKC) system. The different responsiveness of day-4 and day-9 CL to PGE-2 and PGF-2α could depend on receptor availability for these two prostaglandins, even if other cellular mechanisms cannot be excluded. The present study supports a functional role for NOS in regulating the steroidogenic capacity of rabbit CL, and reveals a novel interaction between a stimulatory G-protein-coupled receptor and PKC/PKA-mediated signal transduction modulating NOS activity.

Journal of Endocrinology (2000) **164,** 179–186

Introduction

Since its discovery, nitric oxide (NO), a short-lived radical that transmits signals within and between cells, has been found to exert numerous biological actions, including vasorelaxation, neurotransmission and cytotoxicity, in a variety of physiological and pathophysiological systems (Moncada *et al.* 1991, Snyder & Bredt 1992, Schmidt & Walter 1994). In the past few years, NO has also emerged as a regulator of multiple functions within the reproductive system (Shukovski & Tsafriri 1995, Bonello *et al.* 1996, Yamauchi *et al.* 1997), and several findings suggest that it may have a significant role in the control of steroidogenesis in both ovary and testes (Adams *et al.* 1992, Welch *et al.* 1995, Olson *et al.* 1996, Hesla *et al.* 1997, Jablonka-Shariff & Olson 1997, Pomerantz & Pitelka 1998, Vega *et al.* 1998).

We recently found that constitutive endothelial NO synthase (NOS), a cell-type specific enzyme which catalyses the synthesis of NO, is abundantly expressed in the corpora lutea (CL) of pseudopregnant rabbits (Zampini *et al.* 1998). Subsequent work demonstrated that the NO/NOS system is involved in the regulation of steroidogenesis in rabbit CL and that NO plays a relevant role in the events characterising prostaglandin F-2α (PGF-2α)-induced luteolysis (Gobbetti *et al.* 1999).

The steroidogenic activity and functional life span of CL are controlled by several hormones, which act through luteotrophic and luteolytic stimuli not yet well understood. There is now considerable direct evidence supporting the fact that, in the rabbit, PGF-2α is the major luteolytic hormone (Kehl & Carlson 1981). In several animal species, including man and primates, prostaglandin E-2 (PGE-2) has been identified as a luteotrophic prostaglandin (PG) (Henderson *et al.* 1977, Zelinski-Wooten & Stouffer 1990). Moreover, various reports (Hansel & Dowd 1986, Schlegel *et al.* 1988) have documented that rabbit CL have the capacity to synthesise relatively large amounts of PGs, such as PGE-2 and PGF-2α.

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Prostaglandin-induced complex physiological effects reflect the interaction with cell surface receptors, which influence second messenger systems via G-protein activation (Coleman 1996). It is generally accepted that the actions of these PGs are mediated by protein kinases, but there is still an incomplete understanding of the molecular and cellular regulation of PG-responsive pathways in the CL of the rabbit, as this species has not been as extensively studied as others. Furthermore, no information is available concerning the signalling mechanisms modulating NO production induced by luteotrophic and luteolytic factors after receptor binding, or regulating the functional expression of NOS during the development of the CL in the rabbit.

The present study was designed (1) to determine whether PGE-2 and PGF-2α regulate the activity of luteal NOS differently, and (2) to better elucidate the mechanisms involved in the PG-responsive pathways which control steroidogenesis in isolated CL of pseudopregnant rabbits. With this in view, total NOS activity and progesterone output were monitored after short-term co-incubation of *in vitro* cultured CL obtained on days 4 and 9 of pseudopregnancy with a number of inhibitors of the phospholipase C (PLC)/protein kinase C (PKC), and cAMP/protein kinase A (PKA) systems, together with either PGE-2 or PGF-2α. The study focused on these two sets of CL for their well-established differences *in vivo* (Marcinkiewicz *et al.* 1992, Boiti *et al.* 1998) and *in vitro* responsiveness to PGF-2α (Gobbetti *et al.* 1999).

Materials and Methods

Materials

Medium 199 was obtained from Gibco (Grand Island, NY, USA). Hepes, $NaHCO_3$, BSA, PGF-2α and PGE-2 were purchased from Sigma (St Louis, MO, USA). [1,2,6,7-^{3}H]Progesterone was purchased from Amersham International plc (Amersham, Bucks, UK), while non-radioactive progesterone and progesterone antiserum came from Sigma. Tissue culture plates with 24 flat bottom wells were obtained from Becton Dickinson & Co. (Clifton, NJ, USA). PLC inhibitor, compound 48/80, inositol 1,4,5-triphosphate (InP_3) inhibitor, decavanadate, PKC inhibitor, staurosporine, membrane permeable-adenylate cyclase (AC) inhibitor, 2-*O*-methyladenosine, cell-permeable PKA inhibitor, H89 compound, and NOS inhibitor, N^{ω}-nitro-L-arginine methylester (L-NAME), were all purchased from Sigma. [2,3-^{3}H]L-arginine, having a specific activity of 30–40 Ci/mmol was also from Sigma.

Animals, hormonal regimen and collection of ovaries

For the experiment, 12 mature female New Zealand White rabbits (Charles River Italia, Calco, LC, Italy), 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 serum gonadotrophin (Folligon; Intervet Italia, Milan, Italy) administered i.m., followed 3 days later by an i.m. injection of 0·8 μg gonadotrophin-releasing hormone (GnRH) analogue (Receptal; Roussel-Hoechst, Frankfurt, Germany). Previous experiments in our laboratory showed that this hormonal protocol was effective in inducing ovulation in does (Gobbetti *et al.* 1999). The day of GnRH injection was designated day 0. Rabbits were killed on day 4 (day-4) and on day 9 (day-9) of pseudopregnancy by cervical dislocation. CL were promptly removed from the ovary and collected onto ice-cold medium 199 containing 1% BSA. Isolated CL were then transferred onto filter paper and the non-luteal tissue was dissected away from each CL with fine forceps under stereoscopic magnification. All 4-day-old CL from each rabbit were pooled together, as were those collected from day 9 of pseudopregnancy.

In vitro *incubations*

Corpora lutea, immediately quartered, were randomly distributed (one CL/well) into incubation wells containing 1 ml culture medium 199 with Earles' balanced salt solution containing 2·2 mg/ml sodium bicarbonate, 2·3 mg Hepes and 1% BSA. The incubation set of wells was divided into eight experimental groups. Treatments were (1) control (medium alone), (2) PGF-2α (3 μM), (3) PGF-2α plus PLC inhibitor (1 μg), (4) PGF-2α plus InP_3 inhibitor (1 μg), (5) PGF-2α plus PKC inhibitor (1 μg), (6) PGF-2α plus AC inhibitor (1 μg), (7) PGF-2α plus PKA inhibitor and (8) PGF-2α plus NOS inhibitor (1 μg). Each treatment was performed in four replicate samples using CL obtained from different rabbits. The same experimental scheme was repeated for PGE-2 which was added to the incubation set of wells at a concentration of 3 μM. The culture plates were incubated at 37 °C in air with 5% CO_2. Media were collected 2 h after the addition of treatments and stored immediately at −20 °C for later determination of progesterone. 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. Preliminary evidence led to our choosing the incubation conditions and the minimum effective doses of the substances used in the present *in vitro* study (Fig. 1).

NOS activity determination

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 the NOSdetect Assay Kit (Alexis Corporation, Läufelfingen, Switzerland). The CL from each well were homogenised in 10 vol. (tissue mg/buffer ml) ice-cold homogenisation buffer (250 mM

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Tris–HCl, 10 mM EDTA, 10 mM EGTA, pH 7·4), and centrifuged at 4000 ***g*** for 30 min at 4 °C. Ten microlitres supernatant and 40 µl reaction buffer (50 mM Tris–HCl, 6 µM tetrahydrobiopterin, 2 µM flavin adenine dinucleotide, 2 µM flavin adenine mononucleotide, 10 mM NADPH, 6 mM $CaCl_2$) containing 100 000 d.p.m. [^{3}H]L-arginine were combined. After 30-min incubation at room temperature, the enzymatic reaction was stopped by the addition of 400 µl stop buffer (50 mM Hepes, 5 mM EDTA, pH 5·5). Equilibrated resin (100 µl) was dispensed into the reaction samples, which were transferred to spin cups with cup holders. These were centrifuged in a microcentrifuge at full speed for 30 min, and the elutes were transferred into vials, containing scintillation fluid, to quantify [^{3}H]L-citrulline with the liquid scintillation system LS 1801 (Beckman Instruments, 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).

Progesterone assay

Progesterone concentrations in the incubation medium samples were determined following the RIA previously reported (Gobbetti *et al.* 1992). The assay sensitivity was 10 pg/ml, with a working range of the RIA in culture medium of 1–5 ng/ml. Intra-assay and interassay coefficients of variations were 6% and 11% respectively.

Statistics

The effects of treatments on the release of progesterone and the enzymatic activity of NOS were determined by ANOVA (Sokal & Rohlf 1981), followed by Duncan's multiple range test (Duncan 1955). To normalise the data obtained from different preparations, results are expressed as a mean percentage of basal NOS activity and basal release of progesterone after 2-h *in vitro* incubation with medium alone.

Results

The total NOS activity in rabbit CL cultured with medium alone after 2-h *in vitro* incubation was higher ($P \leq 0{\cdot}01$) on day 4 of pseudopregnancy (18 453 ± 1946 d.p.m./mg wet tissue) than on day 9 (12 634 ± 1938). At the end of the incubation period, the corresponding basal progesterone secretion was lower ($P \leq 0{\cdot}01$) on day-4 CL (3128 ± 168 pg/mg wet tissue) than on day-9 CL (4236 ± 317).

The dose-dependent effects of increasing the concentrations of PGE-2 and PGF-2α added to the incubation medium on both NOS activity and progesterone secretion are shown in Fig. 1. When a response was observed, the NOS activity was inversely related to progesterone release in both PGE-2- and PGF-2α-treated CL.

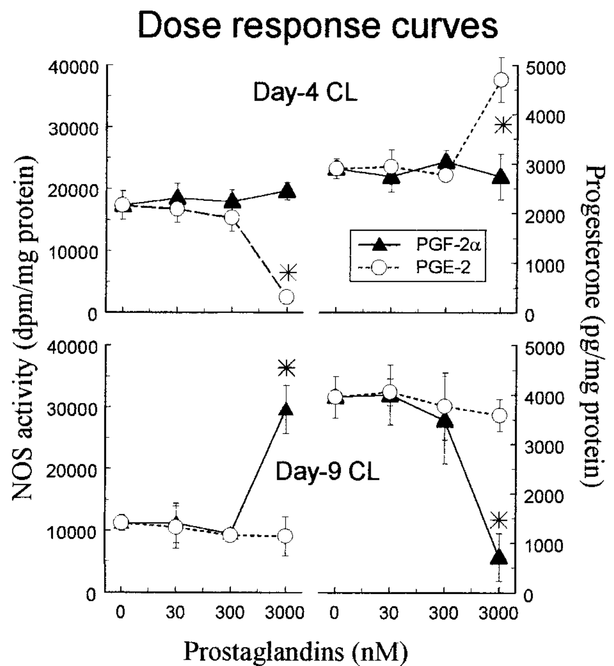

Figure 1 *In vitro* effects of increasing concentrations of PGE-2 and PGF-2α on NOS activity (left-hand side) and progesterone release (right-hand side) by rabbit CL collected on days 4 and 9 of pseudopregnancy and incubated for 2 h. Values are means ± S.D. of three determinations. within each panel, the asterisk indicates significantly different values (*$P \leq 0{\cdot}01$, Duncan's multiple range test with respect to other doses of the same PG).

Effects of PGE-2 on NOS activity and progesterone release by in vitro *cultured CL*

The *in vitro* acute effects of PGE-2 and of PGE-2 co-incubated with either inhibitors of the polyphosphoinositol–PKC second messenger system or inhibitors of the AC/PKA effector system, on NOS activity and progesterone release measured in rabbit CL obtained on days 4 and 9 of pseudopregnancy after 2-h *in vitro* incubation are reported in Fig. 2. On day-4 CL, treatment with PGE-2 significantly ($P \leq 0{\cdot}01$) decreased NOS activity to 13% of control, but increased the release of progesterone ($P \leq 0{\cdot}01$) by 61% over control. By contrast, on day-9 CL both NOS activity and progesterone secretion remained unaffected in the presence of PGE-2 added to the medium.

The co-treatments of 4-day CL tissue preparations with PGE-2 and either inhibitors of PLC or inhibitors of the polyphosphoinositol–PKC second messenger system did not modify the total NOS activity whose values remained very low and similar to those obtained from luteal tissue treated with PGE-2 alone (Fig. 2). The same inhibitors, when applied together with PGE-2 on day-9 CL, did not significantly change the NOS activity, which remained very close to that found in non-stimulated control CL.

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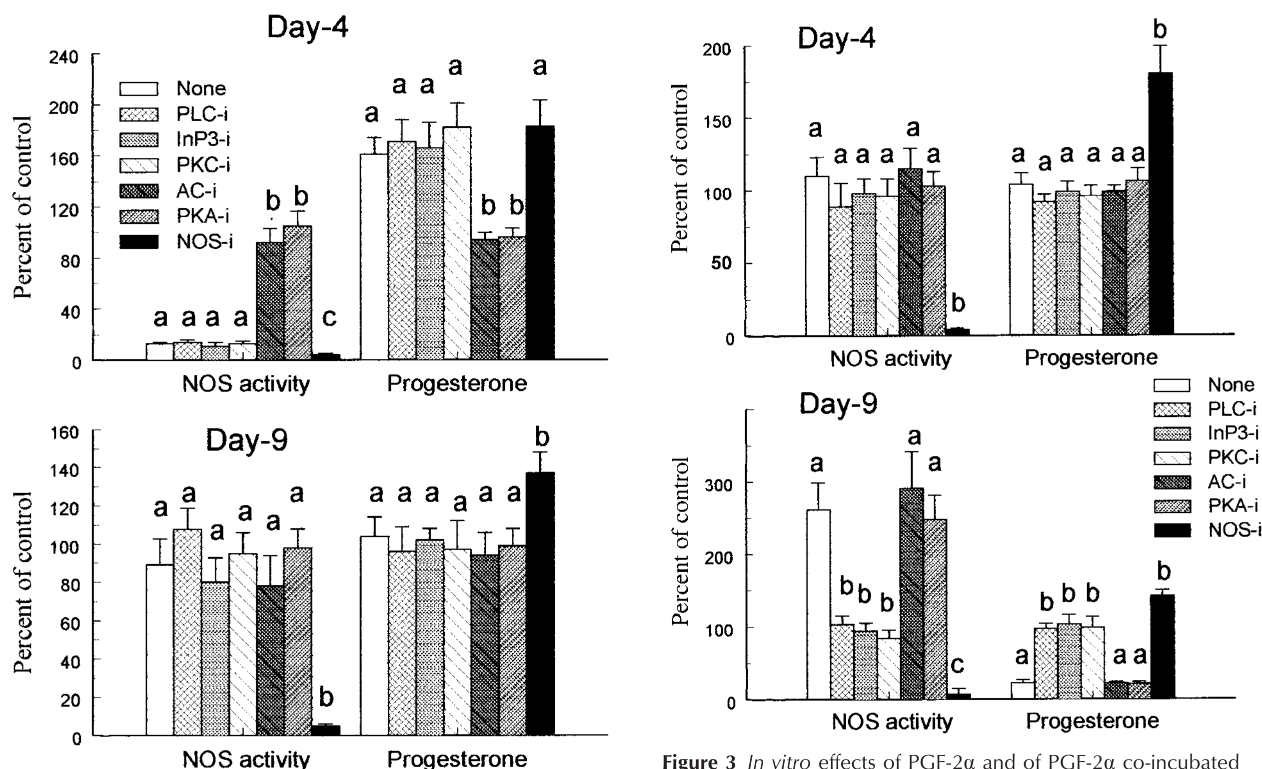

Figure 2 *In vitro* effects of PGE-2 and of PGE-2 co-incubated with different inhibitors, on NOS total activity and progesterone release of rabbit CL collected on days 4 (upper panel) and 9 (lower panel) of pseudopregnancy after 2-h incubation. PLC-i, InP_3-i and PKC-i refer to specific inhibitors of the polyphosphoinositol–PKC second messenger system (compound 48/80, decavanade and staurosporine respectively); AC-i and PKA-i to inhibitors of the cAMP/PKA effector system (2-*O*-methyladenosine and compound H89 respectively); NOS-i to the inhibitor L-NAME (see Materials and Methods for more details). Results are the means ± S.D. of four replicate 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 18 453 ± 1946 and 12 634 ± 1938 d.p.m./mg wet tissue at days 4 and 9 respectively, while those of progesterone production were 3·1 ± 0·2 and 4·2 ± 0·3 ng respectively. Within each panel, different letters above the bars of each series indicate significantly different values ($P \leq 0 \cdot 01$).

Figure 3 *In vitro* effects of PGF-2α and of PGF-2α co-incubated with different inhibitors, on NOS total activity and progesterone release of rabbit CL collected on days 4 (upper panel) and 9 (lower panel) of pseudopregnancy after 2-h incubation. PLC-i, InP_3–i and PKC-i refer to specific inhibitors of the polyphosphoinositol–PKC second messenger system (compound 48/80, decavanade and staurosporine respectively); AC-i and PKA-i to inhibitors of the cAMP/PKA effector system (2-*O*-methyladenosine and compound H89 respectively); NOS-i to the inhibitor L-NAME (see Materials and Methods for more details). Results are the means ± S.D. of four replicate 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 18 453 ± 1946 and 12 634 ± 1938 d.p.m./mg wet tissue at days 4 and 9 respectively, while those of progesterone production were 3·1 ± 0·2 and 4·2 ± 0·3 ng respectively. Within each panel, different letters above the bars of each series indicate significantly different values ($P \leq 0 \cdot 01$).

Similarly, the production of progesterone by either day-4 or day-9 CL was not influenced by co-treatments with these inhibitors, when compared with luteal preparations treated with PGE-2 alone (Fig. 2).

The addition of either AC or PKA inhibitors to PGE-2-treated day-4 CL completely reversed the negative effect induced by PGE-2 alone on NOS, whose total activity rose from 13% to 92% and to 105% of control respectively. They also annulled the steroidogenic effect due to PGE-2, as progesterone release remained at its basal values after 2 h (Fig. 2). Conversely, the same inhibitors of the AC/PKA effector system pathway, when added to day-9 CL, failed to affect both the NOS activity and progesterone release (Fig. 2).

The co-addition of the specific NOS inhibitor, L-NAME (1 μg), to PGE-2-treated wells resulted in a 94–96% inhibition of control NOS basal activity ($P \leq 0 \cdot 01$) and a significant 37–83% increase ($P \leq 0 \cdot 01$) in release of progesterone, independent of the age of the CL (Fig. 2).

Effects of PGF-2α on NOS activity and progesterone release by in vitro *cultured CL*

The *in vitro* effects of PGF-2α and of PGF-2α co-incubated with either inhibitors of polyphosphoinositol–PKC second messenger system or inhibitors of the AC/PKA effector system, on NOS total activity of rabbit CL

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collected on days 4 and 9 of pseudopregnancy after 2-h incubation are shown in Fig. 3.

In day-4 CL, the addition of PGF-2α had no effect on either total NOS activity or progesterone production (Fig. 3). Conversely, in day-9 CL, PGF-2α induced an increase to 261% of NOS baseline activity ($P \leq 0.01$) and a clear luteolytic effect by reducing ($P \leq 0.01$) progesterone release to 23% of control.

The results obtained by the co-treatments of luteal tissues with PGF-2α and the other inhibitors of protein kinases or second messenger systems here tested were almost opposite to those found in the PGE-2 series. In fact, PGF-2α plus either inhibitors of PLC, InP_3 and PKC, or inhibitors of AC and PKA, did not alter either the basal NOS activity or progesterone output found in day-4 CL when compared with control CL of the same age incubated with PGF-2α or medium alone (Fig. 3). By contrast, in day-9 CL, the PLC, InP_3 and PKC inhibitors significantly reduced ($P \leq 0.01$) the NOS activity from 261% found in CL treated with PGF-2α alone, to its basal value observed in non-stimulated control CL. The luteolytic effect induced by PGF-2α on progesterone secretion was also clearly and similarly reversed by co-treatments with these inhibitors.

In day-9 CL, blockage of either AC or the PKA second messenger system by *in vitro* co-treatments of luteal tissue preparations with the specific inhibitors here tested, plus PGF-2α, did not modify the activity of the NOS enzyme, which remained 281% and 248% of control respectively after the same time-interval. These inhibitors had no additive effect on progesterone over that exhibited by the PG itself (Fig. 3).

The co-treatment of L-NAME and PGF-2α similarly reduced ($P \leq 0.01$) basal NOS activity and increased ($P \leq 0.01$) progesterone secretion in the CL of both ages (Fig. 3).

Discussion

This research has revealed that in the rabbit CL both NOS activity and steroidogenesis, as reflected by progesterone production *in vitro*, are modulated by PGE-2 and PGF-2α acutely, but differently, depending on the age of the CL.

Regarding the functional study of NOS, the present data confirm our previous results (Gobbetti *et al.* 1999), as CL collected in the early luteal phase of pseudopregnancy, at day 4, had greater basal activity than did CL collected in the mid-luteal phase, at day 9. Preliminary evidence that rabbit luteal tissue expresses endothelial NOS, which affects progesterone release *in vitro*, together with the present finding that its levels of activity are differently modulated by PGs, strongly supports a functional role for NOS in the regulation of steroidogenic capacity in the CL of this species in response to both luteolytic and luteotrophic factors. In fact, treatment of day-4 CL with PGE-2 increased progesterone output while inhibiting basal NOS activity. On the contrary, in similarly cultured CL collected at day 9 of pseudopregnancy, PGF-2α caused a clear-cut decrease in progesterone production after 2-h incubation and a marked increase of NOS activity. These *in vitro* effects, either luteotrophic or luteolytic, were not only abolished, but also reversed by co-treatment with the NOS inhibitor, L-NAME.

Our study has demonstrated that PGE-2 treatment reduced NOS activity in day-4 CL by activating AC/PKA pathways, implicating an effect of these second messenger systems on the down-regulation of NOS. By contrast, the increased NOS activity induced by the addition of PGF-2α to the *in vitro* cultured CL at day 9 was mediated through PLC/PKC pathways, and probably by an augmented intracellular Ca^{2+} release. So far, even if the involvement of other signal transduction systems cannot be excluded, a key role in the regulation of NOS activity should be attributed to the levels of free intracellular Ca^{2+}. In fact, it is well established that constitutive endothelial 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 by receptors or physical stimulation. Therefore, the known ability of PGF-2α, following receptor binding to target cells, to activate PLC, thereby favouring the formation of InP_3 which, in turn, frees intracellular Ca^{2+}, could explain the activation of NOS to release NO in CL treated with this PG (Veldhuis 1987, Wiltbank *et al.* 1989*b*). The down-regulation of NOS activity induced by PGE-2 in the *in vitro* cultured CL of the early luteal phase could also be attributed to a reduction of intracellular levels of Ca^{2+} in luteal cells (Wiepz *et al.* 1993).

There is now considerable direct evidence suggesting that progesterone production by the rabbit CL *in vitro* is controlled, at least in part, by the NO/NOS system. Although the precise biochemical nature of the control of steroidogenesis by NOS still remains unknown, several suggestions can be offered on the basis of available knowledge. NO may act by selectively inhibiting the cytochrome steroidogenic enzyme $P450_{SCC}$, which controls cholesterol side-chain cleavage in the inner mitochondrial membrane or, more generally, mitochondrial function (Moncada *et al.* 1991). Endogenous NO may also have many other intracellular targets, such as ADP-ribosylating enzymes and the heme-containing enzyme cyclo-oxygenase promoting PG production from arachidonic acid. In cultured human granulosa dispersed cells, NO has been shown to increase intracellular cGMP levels, but the role of this second messenger in the regulation of steroidogenesis is still unclear (Van Voorhis *et al.* 1994).

In the present experiment, treatment with PGF-2α caused a decrease in progesterone secretion by *in vitro* cultured CL of rabbits only at day 9 of pseudopregnancy. By contrast, PGF-2α was totally ineffective on day-4 CL. The suppression of the inhibitory effect on progesterone

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release after co-treatment with selective cell-permeable inhibitors of the PLC/PKC system, would suggest that PGF-2α-induced luteolysis is associated with activation of the PKC pathway and free intracellular Ca^{2+} release also in rabbits, as observed in most species (Davis *et al.* 1987, Wiltbank *et al.* 1989*a*, McGuire *et al.* 1994, Niswender *et al.* 1994). It is widely recognised that PGF-2α acts directly on the CL to inhibit progesterone production with the relevant exception of primates and man, but its effects are closely related to the age of the CL (Behrman *et al.* 1974). Rabbits are completely refractory to PGF-2α injection during both the early and mid-luteal phases until day 12 of pseudopregnancy (Marcinkiewicz *et al.* 1992), even if they exhibit earlier responsiveness to the PGF-2α analogue, alfaprostol (Boiti *et al.* 1998). So far, the intra- and intercellular mechanisms by which PGF-2α exerts its luteolytic action are not yet well understood. Several authors have investigated the responses of steroidogenic luteal cells during PGF-2α-induced luteolysis with regard to intracellular enzymes involved in the regulation of progesterone synthesis (Hawkins *et al.* 1993, McGuire *et al.* 1994, Tian *et al.* 1994, Juengel *et al.* 1995).

Studies of PGF-2α actions *in vitro* have obtained contradictory results on the production of progesterone. McLean *et al.* (1987) found that neither PGE-2 nor PGF-2α, at a concentration of 0·1–3·0 μg/ml, altered progesterone secretion by dispersed luteal cells obtained from rabbits at day 10 of pseudopregnancy. By contrast, O'Grady *et al.* (1972) found a direct inhibition of progesterone production by tissue sections of CL obtained at day 10 of pregnancy and incubated *in vitro* with PGF-2α. Dharmarajan *et al.* (1989) observed that PGF-2α did not affect progesterone in the *in vitro* perfused rabbit ovary at days 11 or 18 of pseudopregnancy. These discrepancies concerning the control of luteal function when applied to *in vitro* studies are probably related to the different techniques used, and also to different physiological conditions between pregnancy and pseudopregnancy. Although several studies indicate that rabbit luteal cells maintain their viability and can be successfully cultured *in vitro*, enzymatic dissociation with collagenase and the culture of dispersed luteal cells might alter the properties of these cells with respect to the responsiveness to luteotrophic or luteolytic hormones (McLean & Miller 1985).

In our *in vitro* model, PGE-2 stimulated progesterone secretion only in day-4 CL, and this effect was abolished by the addition of AC/PKA inhibitors, thus suggesting that this second messenger system is activated by PGE-2. In several species, PGE-2 has been identified as a luteotrophic PG, but the stimulatory actions of PGE-2 on progesterone secretion differ between luteal cell types and the age of CL (Fitz *et al.* 1984, Richards *et al.* 1994). In CL, the effects induced by luteotrophic hormones have been associated with a significant increase in cAMP accumulation in luteal cells (Niswender *et al.* 1994, Rojas & Ciridon 1996, Feng & Almond 1998). Moreover, many of the effects induced by PGF-2α can be antagonised or prevented by PGE-2 both *in vivo* and *in vitro* (Henderson *et al.* 1977, Silvia *et al.* 1984). In the rabbit, however, the biological actions of PGE-2 on CL have not been as extensively investigated as those of PGF-2α. Although, in the rabbit, 17β-oestradiol is recognised as the main luteotrophic hormone (Holt 1989), experimental evidence suggests that in CL of this species potential mechanisms do exist for cAMP-mediated stimulation of progesterone production sensitive to PGE-2 via PKA activation.

In our *in vitro* system, CL collected in the early luteal phase were completely refractory to PGF-2α, while those in mid-phase were totally unresponsive to stimulation by PGE-2, independently of the dosages employed. The reasons for this time-related differential resistance to stimulation by selective PGs are not known, but are probably inherent in the developmental stage of the CL. Several papers have documented that PG multiple biological actions exerted on CL, both luteotrophic and luteolytic, depend upon the availability of functional receptors on target luteal cells, and appear to be mediated through direct stimulatory or inhibitory effects on a number of second messengers following receptor binding. While there is a general agreement about the luteolytic role of PGF-2α, there is still some controversy about the actual cellular mechanisms protecting PG-induced regression of the CL in the early period of pseudopregnancy. The lack of responsiveness of day-4 CL to PGF-2α may be due to the reduced number of functional receptors for this PG or, alternatively, to an increase in the number of receptors for other hormones conveying luteotrophic or antiluteolytic signals. PGs produced locally in the CL may strengthen the response to hormonal stimuli, and thus play a significant role in progesterone secretion at different luteal stages. In the same way, the loss of sensitivity of day-9 CL to PGE-2 could also be ascribed to removal of PGE receptor or to receptor uncoupling from AC. Surprisingly, however, while several studies have identified receptor for both PGE-2 and PGF-2α in luteal cells of different species, similar studies, as far as we know, have not been undertaken in rabbits.

In this study, we measured the changes in total NOS activity and progesterone release induced by PGE-2 and PGF-2α on CL obtained from rabbits on days 4 and 9 of pseudopregnancy and cultured *in vitro*. The present data are consistent with our hypothesis that luteolysis in rabbit CL is evoked by PGF-2α through an increase of NOS activity mediated via PLC and Ca^{2+} mobilisation. The luteotrophic action of PGE-2 in CL at an early stage, mediated via the AC/PKA system, involves a decrease of NOS activity. The actual mechanisms of NO action have not been determined in this luteal tissue and thus the precise function of NOS remains to be studied. The cellular distribution of NOS isoenzymes has not yet been examined in the rabbit CL. The different responsiveness of day-4 and day-9 CL to PGE-2 and PGF-2α could depend

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on receptor availability for these two PGs, even if, to date, other cellular mechanisms cannot be ruled out. Autocrine or paracrine factors produced in response to PGs may contribute to their age-dependent differential refractoriness. Answering these questions may provide new insights into the molecular mechanism controlling the intracellular signalling effectors regulating, on the one hand, progesterone synthesis and release by luteal cells and, on the other, the luteolytic process. The basic question as to whether the effects of NO produced on rabbit CL *in vitro* would also apply *in vivo* under physiological conditions requires further investigation.

In conclusion, all these data strongly support the hypothesis that the *in vitro* luteotrophic or luteolytic effects triggered in rabbit CL by exogenous PGE-2 or PGF-2α are controlled, at least in part, by the NO/NOS system. So far, our results suggest a new opposing intracellular mechanism carried out by PGE-2 and PGF-2α in the regulation of NOS activity and, in turn, progesterone release. This proposed new mechanism could be used by these two PGs in exerting their opposite role in the regulation of CL in rabbits, luteotrophic for PGE-2 and luteolytic for PGF-2α.

Acknowledgements

This work was supported by a grant from Ministero dell' Università e della Ricerca Scientifica e Tecnologica. The authors gratefully acknowledge the revision of the English language text by Dr James Burge of the Linguistic Institute of Camerino University.

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Received 8 June 1999
Accepted 16 September 1999

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