Introduction

Corpus luteum function involves a complex network of regulatory factors affecting steroidogenesis, angiogenesis, remodelling, proteolysis and apoptosis. A growing body of evidence supports an important role for prostaglandins and nitric oxide (NO) in ovarian physiology (Olofsson and Leung, 1994; Van Voorhis et al., 1994). Although progesterone secretion in the luteal phase may be important for the preparation of the endometrium for implantation, NO and prostaglandin E (PGE) could play a regulatory role in some of the major vascular changes, as well as in proteolytic and steroidogenic processes that occur during the formation, maintenance and regression of the corpus luteum (Koos, 1993). It has been demonstrated that the murine and human corpus luteum express the constitutive endothelial NOS (eNOS) and the inducible NOS (iNOS; Van Voorhis et al., 1995; Olson et al., 1996), but not the brain NOS isoform (Jablonka-Shariff and Olson, 1997). Moreover, the expression of iNOS is regulated by gonadotrophins and reaches maximum expression in the corpus luteum 10 days after the injection of hCG (Jablonka-Shariff and Olson, 1997). Although the presence of the COX system, including ligands and receptors in the corpus luteum, is well documented (Feng and Almond, 1996), there is still controversy with regards to the effects of the various prostaglandins on the function of the corpus luteum. PGE may be involved in luteotrophic effects in early corpus luteum formation and maintenance, whereas PGF2α has been shown to have a luteolytic effect (Boiti et al., 2000). Both NO and PGE may be involved in the regulation of the vasculature (Burnett et al., 1992), proteolytic activity (Reich et al., 1991) and steroidogenic activity (Van Voorhis et al., 1994). Several reports demonstrate a complex interaction between the prostaglandin and NO pathways in non-ovarian systems, with particular emphasis on the effect of NO on prostaglandin generation (Di Rosa et al., 1996). In the ovary, NO mediates hCG-induced PGE synthesis in rat luteal cells, which may upregulate progesterone production.

This study explores interactions between the nitric oxide synthase (NOS) and the cyclooxygenase (COX) pathways in the regulation of progesterone production in early corpus luteum cells of rats. Nitric oxide (NO), prostaglandin E (PGE) and progesterone production was analysed in luteal cells of the rat corpus luteum exposed to inhibitors of non-specific NOS, inhibitors of inducible NOS (iNOS) and inhibitors of COX. Equine chorionic gonadotrophin (eCG)/hCG-primed rat corpus luteum cells produced NO, PGE and progesterone in a linear manner during 66 h of culture. Exposure of the cells to the non-specific NOS inhibitor, Nω-nitro-L-arginine (0.15 mmol l⁻¹) for 48 h reduced NO, PGE and progesterone production to 21, 32 and 60% of that of the controls, respectively (P < 0.05 to P < 0.01). Another non-specific NOS inhibitor, Nω-methyl-L-arginine, produced similar inhibitions. Exposure of the cultured cells to S-ethylisothiourea (1 mmol l⁻¹), a selective inhibitor of iNOS, suppressed the production of NO by 63%, PGE by 69% and progesterone by 48%. These findings indicate that production of PGE is regulated partly by iNOS, and that progesterone is probably regulated indirectly by the secondary changes in PGE. The addition of arachidonic acid to Nω-methyl-L-arginine-treated cells resulted in a significant increase in PGE and progesterone production (273 and 186%, respectively) without stimulating NO production. In contrast to the regulation exerted by the NO system on COX activity, the COX system does not modulate NO production in this model. This notion stems from the observation that the COX inhibitors acetylsalicylic acid (5 mmol l⁻¹) and indomethacin (5 µmol l⁻¹) suppressed PGE by 86 and 89%, respectively, and progesterone by 34 and 57%, respectively, but failed to inhibit NO production. The results from the present study indicate that iNOS-mediated NO production is involved in stimulating PGE synthesis in rat luteal cells, which may upregulate progesterone production.
the major steroid product of the corpus luteum. Demonstration of regulatory effects of these systems and their inter-relations may provide pertinent information concerning the mechanisms that govern the function of the corpus luteum.

**Materials and Methods**

**Animals**

Immature female Sabra rats of the Hebrew University strain (Jerusalem) were injected with eCG (15 iu, s.c.) on day 25 and with hCG (10 iu) 50 h later, and then killed after 24 h. Animal maintenance was according to the standards of animal care, and the use of laboratory animals was approved by the Institutional Animal Care and Use Committee (file OPRR-A5011-01).

**Materials**

McCoy’s 5a medium (modified, without serum and supplemented with 2 mmol glutamine l⁻¹, 100 U penicillin ml⁻¹ and 100 mg streptomycin sulphate ml⁻¹) was obtained from Beth Haemek. Trypan blue stain (0.4%, w/v), BSA, deoxyribonuclease (bovine pancreas; 2100 U mg⁻¹), eCG, collagenase 1 (type 1A), indomethacin, N⁶-nitro-L-arginine, N⁴-methyl-L-arginine, PGE, arachidonic acid (pig liver) and anti-serum to PGE were obtained from Sigma Chemicals Co. (St Louis, MO). S-ethylisothiourea was purchased from Tocris Cookson Inc. (Ballwin, MO). hCG was obtained from Organon (Oss) and [H³]PGE2 (100–200 Ci mmol l⁻¹) from Tocris Cookson Inc. (Ballwin, MO). The scintillation fluid was OPTI-FLUOR (Packard).

**In vitro studies**

Corpus luteum cells (5 × 10⁵ viable cells per dish) were obtained from eCG- and hCG-primed rat ovaries, and then cut into small pieces and digested with collagenase–DNase cell dispersal solution as described by Magoffin and Eriksson (1988). This solution contains 0.8% (w/v) collagenase, 2% BSA and 20 μg DNase ml⁻¹, and is diluted 1:1 in McCoy’s medium. The ovarian dispersate was centrifuged at 300 g for 5 min. The cells were washed with McCoy’s medium, centrifuged again and the cell pellet dispersed in the medium. The viable cells were counted and inoculated on to 35 mm × 15 mm tissue culture plastic dishes (Costar, Cambridge, MA) containing 1 ml McCoy’s 5a medium (modified, without serum) supplemented with l-glutamine, penicillin and streptomycin. Cell cultures were maintained for 48 h (or as otherwise stated), without a change of medium, at 37°C under a water-saturated atmosphere of 5% CO₂ and 95% air. At the end of the experiment, the media were collected and stored at −20°C until assayed for progesterone, PGE and NO concentrations. Cell count and viability were estimated at the end of each culture by assay of cell-associated plasminogen activator activity in the culture media, to control for potential deleterious effects of the different inhibitors on the cells in culture. In previous reports it has been established that cell-bound plasminogen activator activity is strongly correlated with viable cell count (Hurwitz et al., 1994). In all experiments, plasminogen activator activity was confirmed to remain within 95–105% of the culture control value (100% activity as measured in the absence of NOS or COX inhibitors).

**Progesterone assay**

Progesterone was assayed by a solid-phase ligand-labelled competitive chemiluminescent immunooassay (Immulite, DPC, Los Angeles, CA). This assay used a calibration range of 0.2–40.0 ng ml⁻¹ and displays high specificity for progesterone. Oestradiol was not detectable at 10 000 ng ml⁻¹. The intra-assay and interassay coefficients of variance at 21 ng progesterone ml⁻¹ were 10.5 (n = 20) and 8.1% (n = 48), respectively, and the detection limit was 0.05 ng ml⁻¹.

**PGE assay**

Determination of PGE concentrations was performed using a radioimmunoassay on the basis of an antibody to PGE produced in rabbits. In brief, 0.5 ml of the specific anti-PGE antibody was added either to 0.1 ml of standards or to 0.1 ml aliquots of the conditioned culture media, and incubated for 30 min at 4°C, followed by the addition of 0.1 ml of [³H]PGE (4500–5500 c.p.m.). After 1 h of incubation at 4°C, the antibody bound-PGE was separated by the addition of 0.2 ml of a mixture of 0.1% (w/v) activated charcoal and 0.1% (w/v) Dextran T-70 in PBS containing 0.15 mol NaCl l⁻¹, 0.1 mol Na₃PO₄ l⁻¹ and 0.1% BSA. After vigorous mixing (vortex), the sample was centrifuged at 1300 g for 10 min at 4°C, the supernatant transferred into scintillation vials to which 7 ml scintillation fluid was added, and the samples counted for 1 min in a beta scintillation counter. PGE was kept as a stock solution of 1 mg ml⁻¹ in absolute ethanol at −70°C. Working dilutions were freshly prepared in PBS containing NaCl, Na₃PO₄ and BSA for standards ranging from 0.15 to 10.0 ng ml⁻¹. The intra-assay and interassay coefficients of variation were 11.2 (n = 28) and 15.7% (n = 12), respectively. According to the manufacturer, crossreactivity at 50% displacement is 100% for PGE₂, 140% for PGE₁, 7.7% for PGF₁α and 6.8% for PGF₂α. The detection limit was 15 pg PGE₂ per tube and the antibody affinity constant Kₐ (Scatchard plot) was 6.3 × 10⁸ l mol⁻¹.

**Determination of nitrite concentrations**

Nitrite, the stable metabolite of NO, was measured by colorimetry using the Griess reaction (Green et al., 1982). A sample (100 μl) was added to an equal volume of the Griess reagent in a microtitre plate. The colour reaction was allowed to develop for 5 min at room temperature after which the absorbance of the sample was recorded at
540 nm. Nitrite concentrations were calculated against a standard curve for sodium nitrite and referred to as NO concentrations. Sensitivity of the Griess reaction (minimal detectable amount) was 1.5 nmol ml−1 and its working range was 1.5–100.0 nmol ml−1. The coefficients of variation for the assay were 8.2 (intra-assay, n = 48) and 17.8% (interassay, n = 17) at 9 ng NO ml−1.

Statistical analysis

All experimental data represent the results obtained from triplicate cultures and are expressed as mean ± se. Each experiment was repeated at least three times. Statistical analysis was performed using the one-sample t test for analysing the significant difference from the control, and ANOVA with the Tukey’s HSD post hoc procedure, as well as the non-parametric Kruskal–Wallis test, for the comparison of different groups (or concentration) for each parameter.

Results

Production of progesterone, NO and PGE by corpus luteum cells: time dependence

Corpus luteum cells (5 × 10^5 cells per dish) were cultured for 22, 44 and 66 h to determine the production of progesterone, NO and PGE in eCG/hCG-primed rat corpus luteum cells. There was a time-dependent and linear increase in progesterone, NO and PGE concentrations, representing a fixed rate of accumulation of the three substances during culture (Fig. 1). After establishing this correlation the following experiments were performed after 48 h of culture.

Effect of non-specific NOS inhibitors on PGE and progesterone accumulation in corpus luteum cells

Corpus luteum cells (5 × 10^5 cells per dish) were cultured in the absence or presence of increasing concentrations (0.05–0.15 mmol l−1) of N^o^-nitro-L-arginine, an established NOS inhibitor, to examine the effect of NO on the COX pathway in eCG/hCG-primed rat ovaries. As expected, corpus luteum cells exposed to N^o^-nitro-L-arginine displayed a significant dose-dependent decrease in nitrite concentration in the culture media (Fig. 2a). The 79%
Inhibition of NO production (at 0.15 mmol l⁻¹) was accompanied by a significant decrease in PGE generation and reached an inhibition of 68% at the same concentration of N⁵-nitro-L-arginine (Fig. 2a). Treatment with N⁵-nitro-L-arginine also significantly reduced progesterone accumulation, but to a lesser extent (40% at 0.15 mmol l⁻¹; Fig. 2a).

Another non-specific NOS inhibitor was tested, to rule out the possibility of substance-specific effects. Treatment with 1 mmol N⁵-nitro-L-arginine l⁻¹ (Fig. 2b) gave essentially similar results, namely marked inhibition of NO generation (75%), suppression of PGE production (48%) and suppression of progesterone accumulation (40%). A one-sample t test was performed for the group of data obtained at 0.1 mmol N⁵-nitro-L-arginine l⁻¹, and indicated significance of the inhibitory effects on all the measured parameters (NO, PGE and progesterone) compared with controls (n = 6, P < 0.05).

Effect of a selective iNOS inhibitor on PGE and progesterone accumulation in corpus luteum cells

Corpus luteum cells (5 × 10⁵ cells per dish) were cultured in the absence or presence of increasing concentrations of S-ethylisothiourea, a potent inhibitor of type II iNOS (Tracey et al., 1995), to examine whether selective inhibition of iNOS has an effect on the COX pathway in eCG/hCG-primed rat ovaries. Corpus luteum cells exposed to S-ethylisothiourea displayed a significant dose-dependent decrease in nitrite concentration in the culture media (Fig. 3). The inhibition of NO production at 1 mmol S-ethylisothiourea l⁻¹ (63%) was accompanied by a significant inhibition of PGE generation (69%). S-ethylisothiourea also significantly reduced progesterone accumulation but to a lesser extent (48%).

Effect of arachidonic acid on PGE and progesterone accumulation in corpus luteum cells pretreated with non-selective NOS inhibitors.

Arachidonic acid was added as a substrate for PGE production to examine whether the inhibition of progesterone accumulation in corpus luteum cells pretreated with NOS inhibitors is due to the attenuation in PGE production. Corpus luteum cells (5 × 10⁵ cells per dish) were cultured with or without 0.1 mmol N⁵-methyl-L-arginine l⁻¹ in the absence or presence of 0.01 mmol arachidonic acid l⁻¹. Corpus luteum cells in the absence of N⁵-methyl-L-arginine displayed a significant stimulation of PGE (Fig. 4b) and a moderate and non-significant stimulation of progesterone production (Fig. 4c). Corpus luteum cells exposed to N⁵-methyl-L-arginine without arachidonic acid displayed a 56% decrease in nitrite concentration in the culture media (Fig. 4a), accompanied by a decrease in PGE generation (35%, Fig. 4b) and a reduced progesterone accumulation (39%, Fig. 4c). However, the addition of 0.01 mmol arachidonic acid l⁻¹ to 0.1 mmol N⁵-methyl-L-arginine l⁻¹ resulted in stimulation of PGE production (273%), accompanied by an increase in progesterone generation (186%), without a significant reduction in NO concentrations. These findings indicate that changes in progesterone concentrations may be due in part to the changes in PGE concentrations.

Effect of COX inhibitors on nitrite and progesterone accumulation in corpus luteum cells

Corpus luteum cells (5 × 10⁵ cells per dish) were cultured in the absence or presence of increasing concentrations (0.5–5.0 mmol l⁻¹) of acetylsalicylic acid, an established COX inhibitor, to examine the reciprocal effect of prostaglandins on the NO pathway in eCG/hCG-primed rat ovaries. As expected, corpus luteum cells exposed to acetylsalicylic acid displayed a dose-dependent decrease of PGE accumulation in the culture media, and reached an inhibition of approximately 86% at concentrations above 1 mmol acetylsalicylic acid l⁻¹. Concomitantly, acetylsalicylic acid decreased progesterone accumulation and reached a maximum inhibition (34%) at 1 mmol acetylsalicylic acid l⁻¹. Treatment with acetylsalicylic acid had no effect on nitrite accumulation (Fig. 5a). Indomethacin, another COX inhibitor, was also used to rule out the possibility of substance-specific effects. Treatment with increasing concentrations (0.5–5.0 mmol l⁻¹) of indomethacin caused attenuation of PGE generation (89%) coupled with a decrease in stimulation of PGE production (273%), accompanied by an increase in progesterone generation (186%), without a significant reduction in NO concentrations. These findings indicate that changes in progesterone concentrations may be due in part to the changes in PGE concentrations.
in progesterone accumulation (57%) at 5 μmol indomethacin l⁻¹. Similar to acetylsalicylic acid, indomethacin had no effect on the NO pathway (Fig. 5b).

Fig. 5. Effect of cyclooxygenase (COX) inhibitors on prostaglandin E (PGE), nitric oxide (NO) and progesterone accumulation in cultures of rat corpus luteum cells. Corpus luteum cells (5 × 10⁵ cells per dish) were obtained and maintained for 48 h in the absence or presence of increasing concentrations of (a) acetylsalicylic acid and (b) indomethacin. Media were assayed for PGE, NO and progesterone. ANOVA analysis confirmed the significance of changes between groups for PGE (F = 56, P = 0.001), NO (F = 11.599, P = 0.001) and progesterone (F = 5.004, P = 0.005). Tukey’s HSD multiple comparisons provided the results indicating the significance of difference of at least P = 0.05: aDifferent from the control group, bDifferent from the AA group and cDifferent from the N⁶-methyl-L-arginine (MLA) group as marked on the appropriate bars.

Discussion

The present study investigated the interaction between the NO and COX pathways and the effect of these pathways on progesterone production in cultures of rat corpus luteum cells. The production of PGE and progesterone was demonstrated to be significantly attenuated by non-selective NOS inhibitors in a time- and dose-dependent manner. Moreover, selective inhibition of iNOS by S-ethylisothiourea had similar, albeit smaller, effects, indicating for the first time that iNOS plays a role in the regulation of PGE and
progesterone production in rat corpus luteum cells. The more pronounced effect of the non-specific NOS inhibitors may reflect the dual inhibition of both the eNOS and iNOS isoforms that are expressed in the corpus luteum.

Arachidonic acid, a precursor of PGE was used to examine whether the attenuation in progesterone production is secondary to the decrease in PGE. The addition of arachidonic acid to cultures of corpus luteum cells pretreated with NOS inhibitors resulted in a significant increase in PGE production concomitant with an increase in progesterone production. These observations indicate that attenuation of progesterone by NOS inhibitors is indirectly correlated with the suppression of PGE production.

The finding from the present study that non-selective NOS inhibitors attenuate prostaglandin production is in agreement with other studies in rat ovarian perfusion (Yamauchi et al., 1997; Motta et al., 1997) and in non-ovarian tissues (Salvemini et al., 1995; Salvemini, 1997). Nevertheless, although it has been documented that iNOS is expressed in the corpus luteum (Van Voorhis et al., 1995; Jablonka-Shariff and Olson, 1997), results of the present study support, for the first time, a functional role for this isoform of NOS in the regulation of PGE in rat corpus luteum.

As both NO and prostaglandins have been implicated as mediators of angiogenesis (Montrucchio et al., 1997), and as the hallmark of early corpora lutea formation is extensive neovascularization, it is possible that NO modulates the COX pathway, which is involved in the regulation of vascular changes in the corpus luteum. The corpus luteum is composed of steroidogenic cells (derived from the granulosa and theca cells) and from a large number of non-steroidogenic cells. The non-steroidogenic cells include endothelial cells and fibroblasts, as well as different sub-populations of leucocytes, comprising 70% of the corpus luteum cells (Vega and Devoto, 1988). PGE may be produced by steroid producing cells, macrophages, endothelial or paraluteal cells (Friden et al., 2000a). Similarly, NO may be produced by endothelial cells and macrophages, and possibly by luteal cells (Vega and Devoto, 1988). Furthermore, Vega and Devoto (1988) demonstrated a close proximity between macrophages and other types of cells in the corpus luteum, and demonstrated the presence of gap junctions between endothelial and steroid cells in the corpus luteum (Grazul-Bilska et al., 1997). Taken together, there is ample evidence to suggest a paracrine effect of NO and PGE on progesterone production in the corpus luteum, involving both accessory (paraluteal) and steroid-producing cells.

Findings from the present study that NOS inhibitors decrease progesterone concentrations and have no apparent effect on the number of cells or oestradiol concentrations corroborate earlier studies showing that NO stimulates progesterone production (Dong et al., 1999; Mitsube et al., 1999). The apparent discrepancy between the findings obtained in whole ovarian cultures in the present study and results from studies that indicate inhibition of progesterone production by NO (Van Voorhis et al., 1995; Olson et al., 1996; Jaroszewski and Hansel, 2000) may be explained in part by the need to preserve the cellular compartments to allow cell–cell interaction (Dong et al., 1999; Mitsube et al., 1999). Nonetheless, considering the complexity of this system, it is not possible to explain the differences in progesterone response observed in the various experimental models. It was demonstrated that NO decreases progesterone production in the human corpus luteum in the late- but not in the mid-luteal phase (Friden et al., 2000b), implying varied effects of NO during the corpus luteum lifespan, that is luteotrophic in the early and luteolytic in the late luteal phase.

The results of the present study also indicate that in this experimental model the accumulation of NO is not affected by the COX inhibitors indomethacin and acetylsalicylic acid. Although this finding substantiates similar effects (in non-ovarian tissue) of indomethacin (Di Rosa et al., 1996), other studies (Amin et al., 1995) noted that acetylsalicylic acid inhibits the induction of NOS, probably through a mechanism independent of prostaglandins.

Collectively, findings from the present study and observations on corpus luteum of other species (Tanaka et al., 1991; Fulghesu et al., 1993) indicate that prostaglandins do not serve as obligatory mediators of NO biosynthesis in corpus luteum cells, but seem to have a direct role in the regulation of progesterone production.

In conclusion, the present study is the first to report that iNOS is involved in the downstream ‘chain of command’ regulating PGE and progesterone production in the corpus luteum. These findings imply that availability of NO produced by iNOS is essential for unimpaired PGE and progesterone accumulation in the corpus luteum cells.

References


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