Regulation of cyclooxygenase activity and progesterone production in the rat corpus luteum by inducible nitric oxide synthase

A. Hurwitz¹, Z. Finci-Yeheskel², A. Milwidsky¹ and M. Mayer²

Departments of ¹Obstetrics and Gynecology and ²Clinical Biochemistry, Hadassah University Hospital, Mt Scopus, Jerusalem, IL-91240, Israel

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, *N*^ω-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, *N*^ω-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 *N*^ω-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.

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, both 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 PGF_{2α} 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 generation by oocyte–cumulus complexes (Jawerbaum *et al.*, 1997).

Given the established expression of COX and NOS in luteal cells and their potential importance, the present study was undertaken to examine the interactions between NO and PGE in the early phase of the rat corpus luteum, and their involvement in the control of progesterone production,

Email: hurwitz@cc.huji.ac.il

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, N^{ω} -nitro-L-arginine, N^{ω} -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 [^3H]PGE₂ (100–200 Ci mmol⁻¹) from DuPont (NEN, Boston, MA). The scintillation fluid was OPTI-FLUOR (Packard).

In vitro studies

Corpus luteum cells (5×10^5 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 $\times$ 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 immunoassay (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 NaN₃ 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[–], NaN₃[–] 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 PGF_{1 α} and 6.8% for PGF_{2 α} . The detection limit was 15 pg PGE₂ per tube and the antibody affinity constant K_a (Scatchard plot) was $6.3 \times 10^9 \text{ l mol}^{-1}$.

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

Fig. 1. Production of nitric oxide (NO), prostaglandin E (PGE) and progesterone as a function of time in cultures of rat corpus luteum cells. Corpus luteum cells (5×10^5 cells per dish) obtained from equine chorionic gonadotrophin (eCG)/hCG-primed rats were cultured for 22, 44 and 66 h under serum-free conditions. NO (■), PGE (▲) and progesterone (●) concentrations in the spent media were measured. Results are mean $\pm$ SE ($n = 4$).

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 $1.5\text{--}100.0 \text{ nmol ml}^{-1}$. 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 $\pm$ 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.

Fig. 2. Effect of increasing concentrations of the nitric oxide synthase (NOS) inhibitors (a) *N*^ω-nitro-L-arginine (NNLA) and (b) *N*^ω-methyl-L-arginine (MLA) on nitric oxide (NO, ■), prostaglandin E (PGE, ▲) and progesterone (●) accumulation in cultures of rat corpus luteum cells. Corpus luteum cells (5×10^5 cells per dish) were obtained and maintained for 48 h with increasing concentrations of the inhibitors. Media were assayed for NO, PGE and progesterone. The 100% values for the data were (a) NO, $5.8 \pm 1.4 \text{ nmol ml}^{-1}$; PGE, $23.6 \pm 2.5 \text{ ng ml}^{-1}$; progesterone, $47 \pm 2 \text{ ng ml}^{-1}$ (mean $\pm$ SE, $n = 6$); and (b) NO, $4.0 \pm 0.3 \text{ nmol ml}^{-1}$; PGE, $50 \pm 5 \text{ ng ml}^{-1}$; progesterone, $53 \pm 5 \text{ ng ml}^{-1}$ (mean $\pm$ SE, $n = 6$). *A *t* test confirmed the significance of the change from control values ($P < 0.05$, $n = 6$).

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 ($0.05\text{--}0.15 \text{ mmol l}^{-1}$) of *N*^ω-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*^ω-nitro-L-arginine displayed a significant dose-dependent decrease in nitrite concentration in the culture media (Fig. 2a). The 79%

Fig. 3. Effect of increasing concentrations of the specific inducible nitric oxide synthase (iNOS) inhibitor *S*-ethylisothiourea (EIT) on nitric oxide (NO, ■), prostaglandin E (PGE, ▲) and progesterone (●) accumulation in cultures of rat corpus luteum cells. Corpus luteum cells (5×10^5 cells per dish) were obtained and maintained for 48 h with increasing concentrations of EIT. Media were assayed for NO, PGE and progesterone. The 100% values were (a) NO, 5.8 ± 1.4 nmol ml⁻¹; PGE, 24 ± 3 ng ml⁻¹; progesterone, 44 ± 3 ng ml⁻¹ (mean $\pm$ SE, $n = 6$) after ANOVA analysis, which confirmed the significance of the differences between groups for NO ($F = 4.860$, $P = 0.009$), PGE ($F = 3.232$, $P = 0.044$) and progesterone ($F = 17.045$, $P = 0.000$). Kruskal–Wallis test showed significance of 0.020, 0.083 and 0.001 for the effects of EIT on NO, PGE and progesterone concentrations, respectively. *Indicates significant change from the control values ($P < 0.01$).

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 *N*^ω-nitro-L-arginine (Fig. 2a). Treatment with *N*^ω-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 *N*^ω-methyl-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 *N*^ω-methyl-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 (5×10^5 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 (5×10^5 cells per dish) were cultured with or without 0.1 mmol *N*^ω-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 *N*^ω-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 *N*^ω-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 *N*^ω-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 (5×10^5 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 μmol l⁻¹) of indomethacin caused attenuation of PGE generation (89%) coupled with a decrease

Fig. 4. Effect of arachidonic acid (AA) on (a) nitric oxide (NO), (b) prostaglandin E (PGE) and (c) progesterone accumulation in cultures of rat corpus luteum cells. Corpus luteum cells (5×10^5 cells per dish) were obtained and maintained for 48 h in the absence or presence of increasing concentrations of AA. 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 N^w-methyl-L-arginine (MLA) group as marked on the appropriate bars.

in progesterone accumulation (57%) at 5 μ mol 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 (5×10^5 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 (●). The 100% values were (a) PGE, 130 ± 5 ng ml⁻¹; NO, 12.7 ± 3.0 nmol ml⁻¹; and progesterone, 37 ± 5 ng ml⁻¹ (mean $\pm$ SE, $n = 6$), and (b) PGE, 70 ± 12 ng ml⁻¹; NO, 12.8 ± 3.0 nmol ml⁻¹; progesterone, 26 ± 7 ng ml⁻¹ (mean $\pm$ SEM, $n = 6$). *A compilation of results from this and additional experiments ($n = 7$) showed that the concentrations of PGE and progesterone in presence of 1 mmol acetylsalicylic acid l⁻¹ or 1 μ mol indomethacin l⁻¹ are significantly different from controls ($P < 0.01$).

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 subpopulations 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 support 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 concentration 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

- Amin AR, Vyas P, Attur M, Leszczynska-Piziak J, Patel IR, Weissmann G and Abramson SB (1995) The mode of action of acetylsalicylic acid-like drugs: effect on inducible nitric oxide synthase *Proceedings National Academy of Sciences USA* **92** 7926–7930
- Boiti C, Zerani M, Zampini D and Gobetti A (2000) Nitric oxide synthase activity and progesterone release by isolated corpora lutea of rabbits in the early- and mid-luteal phases of pseudopregnancy are modulated differentially by prostaglandin E₂ and prostaglandin F_{2α} via adenylyl cyclase and phospholipase C *Journal of Endocrinology* **164** 179–186
- Burnett AL, Lowenstein CJ, Brecht DS, Chang TS and Snyder SH (1992) Nitric oxide: a physiologic mediator of penile erection *Science* **257** 401–403
- Di Rosa M, Ialenti A, Iannaro A and Sautebin L (1996) Interaction between nitric oxide and cyclooxygenase pathways *Prostaglandins, Leukotrienes and Essential Fatty Acids* **54** 229–238
- Dong YL, Gangula PRR and Fang L (1999) Nitric oxide reverses the prostaglandin-induced inhibition of progesterone secretion in rats *Human Reproduction* **14** 27–32
- Feng SM and Almond GW (1996) Identification and distribution of prostaglandin E receptors on porcine luteal cells *Biology of Reproduction* **54** 1366–1374
- Friden BE, Runesson E, Hahlin M and Brannstrom M (2000a) Evidence for nitric oxide acting as a luteolytic factor in human corpus luteum *Molecular Human Reproduction* **5** 397–403
- Friden BE, Wallin A and Brannstrom M (2000b) Phase-dependent influence

- of non-steroidogenic cells on steroidogenesis and prostaglandin production by the human corpus luteum *Fertility and Sterility* **73** 359–365
- Fulghesu AM, Lanzone A, Di Simone N, Nicoletti MC, Caruso A and Mancuso S (1993) Indomethacin *in vivo* inhibits the enhancement of the progesterone secretion in response to gonadotropin-releasing hormone by human corpus luteum *Human Reproduction* **8** 35–39
- Grazul-Bilska AT, Redmer DA and Reynolds LP (1997) Cellular interactions in the corpus luteum *Seminars in Reproductive Endocrinology* **15** 383–393
- Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS and Tannenbaum SR (1982) Analysis of nitrate, nitrite and ¹⁵N nitrate in biological fluids *Analytical Biochemistry* **126** 131–158
- Hurwitz A, Finci-Yeheskel Z, Dushnik M, Milwidsky A, Ben-Chetrit A, Yagel S, Adashi EY and Mayer M (1994) Cytokine-mediated regulation of rat ovarian function. Interleukin-1 inhibits plasminogen activator activity through the induction of plasminogen activator inhibitor 1 (PAI 1) *Molecular and Cellular Endocrinology* **101** 307–314
- Jablonka-Shariff A and Olson LM (1997) Hormonal regulation of nitric oxide synthases and their cell-specific expression during follicular development in rat ovary *Endocrinology* **138** 460–468
- Jaroszewski JJ and Hansel W (2000) Intraluteal administration of a nitric oxide synthase blocker stimulates progesterone and oxytocin secretion and prolongs the life span of the bovine corpus luteum *Proceedings – Society for Experimental Biology and Medicine* **224** 50–55
- Jawerbaum A, Gonzalez ET, Faletti A, Novaro V and Gimeno MA (1997) Nitric oxide mediates human chorionic gonadotropin-induced prostaglandin E generation in rat oocyte–cumulus complexes *Reproduction, Fertility and Development* **9** 391–394
- Koos RD (1993) Ovarian angiogenesis. In *The Ovary* pp 433–453 Eds EY Adashi and PCK Leung. Raven Press, New York
- Magoffin DA and Eriksson GF (1988) Improved method for primary cultures of ovarian androgen producing cells in serum free medium: effect of lipoproteins, insulin and insulin-like growth factor *in vivo*. *In Vitro Cellular and Developmental Biology* **24** 862–870
- Mitsube K, Mikuni M, Matousek M and Brannstrom M (1999) Effects of a nitric oxide donor and nitric oxide synthase inhibitors on luteinizing hormone-induced ovulation *in vivo* perfused rat ovary *Human Reproduction* **14** 2537–2543
- Montrucchio G, Lupia E, de Martino A, Battaglia E, Arese M, Tizzani A, Bussolino F and Camussi G (1997) Nitric oxide mediates angiogenesis induced *in vivo* by platelet-activating factor and tumor necrosis alpha *American Journal of Pathology* **151** 557–563
- Motta AB, Franchi AM and Gimeno MF (1997) Role of nitric oxide on uterine and ovarian prostaglandin synthesis during luteolysis in the rat *Prostaglandins, Leukotrienes and Essential Fatty Acids* **56** 265–269
- Olofsson J and Leung PC (1994) Auto/paracrine role of prostaglandins in corpus luteum function *Molecular and Cellular Endocrinology* **100** 87–91
- Olson LM, Jones-Burton CM and Jablonka-Shariff A (1996) Nitric oxide decreases estradiol synthesis of rat luteinized ovarian cells: possible role for nitric oxide in functional luteal regression *Endocrinology* **137** 3531–3539
- Reich R, Daphna-Iken D, Chun SY, Popliker M, Slager R, Adelman-Grill BC and Tsafiri A (1991) Preovulatory changes in ovarian expression of collagenases and tissue metalloproteinase inhibitor messenger ribonucleic acid: role of eicosanoids *Endocrinology* **129** 1869–1875
- Salvemini D (1997) Regulation of cyclooxygenase enzymes by nitric oxide *Cellular and Molecular Life Sciences* **53** 576–582
- Salvemini D, Manning PT, Zweifel BS, Seibert K, Connor J, Currie MG, Needleman P and Masferrer JL (1995) Dual inhibition of nitric oxide and prostaglandin production contributes to the anti-inflammatory properties of nitric oxide synthase inhibitors *Journal of Clinical Investigation* **96** 310–308
- Tanaka N, Espey LL, Kawano T and Okamura H (1991) Comparison of inhibitory actions of indomethacin and epistane on ovulation in rats *American Journal of Physiology* **260** E170–174
- Tracey WR, Nakane MN, Basha F and Carter G (1995) *In vivo* pharmacological evaluation of two novel type II (inducible) nitric oxide synthase inhibitors *Canadian Journal of Physiology and Pharmacology* **73** 665–669
- Van Voorhis BJ, Dunn MS, Snyder GD and Weiner CP (1994) Nitric oxide: an autocrine regulator of human granulosa luteal cell steroidogenesis *Endocrinology* **135** 1799–1806
- Van Voorhis BJ, Moore K, Strijbos PJ, Nelson S, Baylis SA, Grzybicki D and Weiner CP (1995) Expression and localization of inducible and endothelial nitric oxide synthase in the rat ovary. Effects of gonadotropin stimulation *in vivo*. *Journal of Clinical Investigation* **96** 2719–2726
- Vega M and Devoto L (1988) Autocrine/paracrine regulation of normal human corpus luteum development *Seminars in Reproductive Endocrinology* **15** 353–362
- Yamauchi J, Miyazaki T, Iwasaki S, Kishi I, Kuroshima M, Tei C and Yoshimura Y (1997) Effect of nitric oxide on ovulation and ovarian steroidogenesis and prostaglandin production in the rabbit *Endocrinology* **138** 3630–3637

Received 19 September 2001.

First decision 8 November 2001.

Revision received 17 December 2001.

Accepted 10 January 2002.