Relationship between the production of prostaglandins and progesterone by luteinizing human granulosa cells

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Abstract

Luteinizing granulosa cells synthesize high concentrations of progesterone, prostaglandin (PG) E₂ and PGF_{2α}. The objective of this study was to explore the relationship between prostaglandin and progesterone output from human granulosa cells as they undergo functional luteinization in culture. Granulosa cells were partially purified from ovarian follicular aspirates and cultured at a density of 10⁵ cells/ml in serum-supplemented DMEM:Ham's F₁₂ medium for 0, 1 or 2 days. Cells were then switched to serum-free medium for 24 h before measuring hormone concentrations in this spent medium by specific radioimmunoassays. Over the first 3 days in culture, PGF_{2α} and PGE₂ production declined progressively by up to 82 ± 3% coincident with a 55 ± 11% increase in progesterone

output. In subsequent experiments, cells were treated for 24 h on the second day of culture with either 0.01 to 10 μM meclofenamic acid or with 10 μM and 100 μM aminoglutethimide. Meclofenamic acid inhibited synthesis of PGF_{2α} and PGE₂ by up to 70 ± 9% and 64 ± 7% respectively without affecting progesterone output. Likewise, 100 μM aminoglutethimide inhibited progesterone production by 62 ± 6% without affecting concentrations of either PGF_{2α} or PGE₂. We have concluded that the progressive decline in prostaglandin production and the rise in progesterone output from luteinizing human granulosa cells occur independently of each other.

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Introduction

Following the preovulatory gonadotrophin surge, induction of the expression of cyclo-oxygenase/prostaglandin H synthase (PGHS)-2 confers on luteinizing granulosa cells the capacity to synthesize high concentrations of prostaglandins (LeMaire *et al.* 1975, Espey 1980, Ainsworth *et al.* 1984, Richards 1994, Sirois 1994, Narko *et al.* 1997, Richards *et al.* 1998). Increased generation of these inflammatory mediators has been implicated in follicular rupture and ovulation of the mature oocyte (Grinwich *et al.* 1972, Espey 1980, Richardson 1986, Munalulu *et al.* 1987). However, the significance of locally synthesized prostaglandins in the paracrine/autocrine control of ovarian steroidogenesis has yet to be established. A number of studies in a wide range of species have demonstrated that treatment of luteinized cells with exogenous prostaglandin (PG) E₂ stimulates progesterone biosynthesis whereas PGF_{2α} and its analogues have generally been

reported to inhibit luteal steroidogenesis (reviewed in Richardson 1986, Michael *et al.* 1993, 1994, Olofsson & Leung 1994). Based on these documented effects, we hypothesized that locally synthesized prostaglandins may act in an autocrine/paracrine manner to dictate the level of progesterone synthesis in luteinizing granulosa cells. Moreover, since progesterone has been inversely correlated with prostaglandin synthesis in the corpus luteum (CL) (Patek & Watson 1976, Rothchild 1981), we also speculated that increasing output of progesterone from luteinizing granulosa cells may suppress PGF_{2α} and/or PGE₂ production from such cells. Hence, the objective of the present study was to investigate the relationship between the production of both PGF_{2α} and PGE₂ and the output of progesterone from human granulosa cells as they luteinize *in vitro*. To examine the role of endogenous prostaglandins in regulating progesterone synthesis, cells were treated with meclofenamic acid, a pharmacological inhibitor of prostaglandin biosynthesis (Boctor *et al.* 1986,

Zelinski-Wooten *et al.* 1990). Similarly, in order to establish the role of progesterone in the paracrine control of prostaglandin production, endogenous progesterone synthesis was suppressed by treatment of cells with aminoglutethimide, a known inhibitor of steroidogenic cytochrome P450 enzymes (Wilroy *et al.* 1968, Touitou *et al.* 1973, El-Hefnawy & Huhtaniemi 1998, Peluso & Pappalardo 1999).

Materials and Methods

Materials

Phosphate-buffered saline (PBS), Earle's balanced salt solution (EBSS), 1:1 (v/v) Dulbecco's modified Eagle's medium:Ham's F₁₂ (DMEM:F₁₂) medium, foetal calf serum (FCS), antibiotics and L-glutamine were all purchased from Life Technologies Ltd (Uxbridge, Middx, UK). Organic solvents, which were of HPLC grade, were supplied by Merck (Poole, Dorset, UK). Percoll and all other compounds were purchased from Sigma Chemical Co. (Poole, Dorset, UK). Antiserum for the immunoassay of progesterone was obtained from the Central Veterinary Laboratory (Weybridge, Surrey, UK) while antibodies to PGE₂ and PGF_{2α} were generously donated by Drs R W Kelly and N L Poyser respectively (University of Edinburgh, Edinburgh, UK).

Patient samples

Ovarian follicular aspirates were collected from women undergoing assisted conception at the Hallam Medical Centre, Harley Street, London, UK with informed patient consent (in accordance with the Declaration of Helsinki) and with the approval of the local ethics committee. Pituitary down-regulation was achieved by the subcutaneous administration of a gonadotrophin-releasing hormone (GnRH) analogue (Suprecur; Shire Pharmaceuticals, Andover, Hants, UK; 500 µg/day from day 2 of the cycle for 10–21 days). Administration of the GnRH analogue was then continued in conjunction with purified urinary human menopausal gonadotrophin (Menogon; Ferring Pharmaceuticals, Feltham, Middx, UK; 2–4 ampoules daily for 10–14 days) followed by a single intramuscular injection of human chorionic gonadotrophin (Profasi; Serono, Welwyn Garden City, Herts, UK; 5000–10 000 IU) 36 h prior to oocyte collection. Follicles were subsequently aspirated under local anaesthesia by the transvaginal route.

Isolation of human granulosa-lutein cells

Ovarian follicular aspirates were kept at room temperature and were sent to the Royal Free Campus of the Royal Free and University College Medical School within 3 h of

collection. On arrival, ovarian cells and contaminating blood cells were precipitated from the supernatant follicular fluid and follicular flushing medium (EBSS) by centrifugation at 250 g for 10 min at 4 °C. After resuspension in PBS, human granulosa cells were partially purified on 60% (v/v) Percoll as described previously (Webley *et al.* 1988). The number of granulosa cells obtained from a given patient using this protocol ranged between 1.4 and 7.6 × 10⁶ cells. Cell viabilities, which were assessed by the exclusion of trypan blue dye, were routinely in excess of 85% and did not change significantly over the course of any of the experiments described below.

Culture of human granulosa-lutein cells: collection of spent culture medium for hormone assays

In all experiments, cells were seeded into sterile 24-well cell culture plates at a density of 1 × 10⁵ viable cells/ml culture medium with a volume of 1 ml medium per well. In cases where prostaglandin and progesterone production were to be estimated over the first 24 h of culture, cells were seeded in DMEM:Ham's F₁₂ medium supplemented with 0.01% (w/v) bovine serum albumin (BSA), penicillin (87 000 IU/l), streptomycin (87 mg/l) and L-glutamine (2 mM). In all other experiments, cells were prepared and seeded in the same cell culture medium supplemented with 10% (v/v) FCS in place of the BSA. Cells were subsequently incubated at 37 °C in a humidified atmosphere of 5% (v/v) CO₂ in air.

For daily measurements of progesterone and prostaglandin concentrations, cells were incubated for 0, 1 or 2 days in serum-supplemented medium before being switched to serum-free medium for a period of 24 h (designated as day 1, 2 or 3 of cell culture respectively). To remove any residual serum, wells were each rinsed twice with 200–300 µl warmed serum-free medium and this washing medium discarded before adding 1 ml fresh serum-free medium to each well. At the end of the 24-h collection period, the spent serum-free medium was transferred to micro test tubes and stored frozen at –20 °C pending progesterone, PGE₂ and PGF_{2α} assays. (Analysis by light microscopy confirmed that this spent culture medium did not contain detached granulosa-lutein cells.)

Effects of meclofenamic acid on prostaglandin and progesterone production

Cells were cultured overnight in serum-supplemented medium to allow the cells to attach to the wells of the cell culture plate. On the second day of culture, cells were rinsed with serum-free medium and then incubated for a further 24 h in serum-free medium containing 0, 0.01, 0.1, 1 or 10 µM meclofenamic acid. (These concentrations were selected based on an IC₅₀ for the inhibition of PGHS activity by meclofenamic acid of 0.6 µM (Boctor *et al.* 1986).) At the end of the 24-h incubation period, medium

was collected and stored at -20°C pending assay of the prostaglandin and progesterone concentrations. Meclofenamic acid was prepared to a stock concentration of 100 mM in dimethylsulphoxide, the final concentration of which was adjusted in all wells to 0.1% (v/v).

Effects of aminoglutethimide on prostaglandin and progesterone production

After an overnight culture in serum-supplemented medium, cells were rinsed with serum-free medium and then incubated for a further 24 h in serum-free medium containing aminoglutethimide at concentrations of 0, 10 or 100 μM . (These concentrations were selected on the basis of previous reports demonstrating inhibition of adrenal and gonadal steroidogenesis at aminoglutethimide concentrations of 86–500 μM (Wilroy *et al.* 1968, Touitou *et al.* 1973, El-Hefnawy & Huhtaniemi 1998, Peluso & Pappalardo 1999).) At the end of the 24-h incubation period, medium was collected and stored at -20°C pending assay of the prostaglandin and progesterone concentrations. A stock concentration of 100 mM aminoglutethimide was prepared in chloroform. The final concentration of this organic solvent was adjusted in all wells to 0.1% (v/v).

Hormone assays

All hormone concentrations were measured in 100 μl aliquots of serum-free culture medium using radioimmunoassay (RIA) protocols that have been described previously (Kelly *et al.* 1986, Poyser 1987, Pallikaros *et al.* 1995). For each RIA, samples were assayed at dilutions of up to 1/1000 as required to ensure that the hormone measurements were made within the linear range of the relevant standard curve. In our hands, the progesterone RIA was found to have a working range of 0.4–15 nM with intra- and interassay coefficients of variation (CV) of 8% and 14% respectively. The working ranges for the $\text{PGF}_{2\alpha}$ and PGE_2 assays were equal to 1.6–28 nM and 0.1–7 nM respectively. The intra- and interassay CV values were less than 8% and 11% respectively for both prostaglandin assays. The antibody used in the RIA for PGE_2 showed a cross-reactivity of 0.47% for $\text{PGF}_{2\alpha}$ at 50% binding and the antibody used in the $\text{PGF}_{2\alpha}$ RIA exhibited 0.17% cross-reactivity with PGE_2 .

Statistics

All data were subjected to statistical analysis using GraphPad Prism 2 software (San Diego, CA, USA). The time-dependent changes in prostaglandin and progesterone output from cultured cells were evaluated in the first instance by one-way ANOVA with repeated measures (across individual experiments) followed by Dunnett's multiple comparison considering hormone concentrations

on the first day of culture as control values. All concentration-dependent effects of a given treatment on the concentrations of progesterone and/or prostaglandins were similarly assessed by one-way ANOVA with repeated measures (for cells from a given patient) followed, as appropriate, by Dunnett's test. Probabilities of less than 0.05 were accepted as statistically significant in all analyses. All experiments were repeated three to twelve times with triplicate wells for each condition in each experiment. A given experiment featured granulosa-lutein cells from one patient only and, for each patient, cells were pooled from all aspirated follicles.

Results

Daily patterns of prostaglandin and progesterone production

Over the first 3 days of culture, concentrations of both $\text{PGF}_{2\alpha}$ and PGE_2 declined progressively by $82 \pm 3\%$ and $75 \pm 8\%$ respectively (means $\pm$ s.e. of nine independent experiments; $P < 0.05$ relative to corresponding prostaglandin concentrations on the first day of culture; Fig. 1A and B). Over this same culture period, the concentration of progesterone increased by $55 \pm 11\%$ (mean $\pm$ s.e. of twelve independent experiments) relative to the progesterone concentrations on the first day of culture (Fig. 1C). The progressive rise in progesterone production over the first 3 days in culture is in agreement with several previous studies into the endocrinology of human granulosa cells as they undergo functional luteinization *in vitro* (e.g. McNatty *et al.* 1979, Polan *et al.* 1984, Hurwitz *et al.* 1987, Tapanainen *et al.* 1987, Wickings *et al.* 1989, Michael & Webley 1991, Brzezinski *et al.* 1992).

Effects of meclofenamic acid on prostaglandin and progesterone production

Incubation for 24 h with meclofenamic acid resulted in a concentration-dependent suppression of $\text{PGF}_{2\alpha}$ production at concentrations of meclofenamic acid ≥ 10 nM (Fig. 2A). At the maximum tested concentration of 10 μM , meclofenamic acid suppressed $\text{PGF}_{2\alpha}$ production by $70 \pm 10\%$ (mean $\pm$ s.e. of five independent experiments; $P < 0.01$). At concentrations of 1 μM and 10 μM , meclofenamic acid also inhibited PGE_2 accumulation by $73 \pm 6\%$ and $71 \pm 8\%$ respectively (means $\pm$ s.e. of five independent experiments; $P < 0.05$; Fig. 2B). However, progesterone synthesis was unaffected by meclofenamic acid at any of the tested concentrations (Fig. 2C).

Effects of aminoglutethimide on prostaglandin and progesterone production

Incubation for 24 h with 100 μM aminoglutethimide suppressed progesterone production by $64 \pm 7\%$ (mean $\pm$ s.e.

Figure 1 Hormone concentrations in spent culture medium collected over 24-h intervals during continuous culture of human granulosa-lutein cells. Values are the means $\pm$ S.E. for nine ((A) PGF_{2α} and (B) PGE₂) or 12 ((C) progesterone) independent experiments with triplicate determinations in each experiment. * $P < 0.05$, ** $P < 0.01$ relative to concentrations of the appropriate hormone on day 1 of culture.

Figure 2 Concentration-dependent effects of meclofenamic acid on the accumulation of (A) PGF_{2α}, (B) PGE₂ and (C) progesterone in spent medium from cultured human granulosa-lutein cells. All values are the means $\pm$ S.E. for five independent experiments with triplicate determinations in each experiment. * $P < 0.05$, ** $P < 0.01$ versus control.

Figure 3 Concentration-dependent effects of aminoglutethimide on the accumulation of (A) PGF_{2α}, (B) PGE₂ and (C) progesterone in spent medium from cultured human granulosa-lutein cells. Values are the means $\pm$ S.E. for three (PGF_{2α}) or five (PGE₂ and progesterone) independent experiments with triplicate determinations in each experiment. ** $P < 0.01$ versus control.

of five independent experiments; $P < 0.01$; Fig. 3C) with no significant effect at 10 μ M aminoglutethimide. However, treatment with an effective concentration of this

cytochrome P450 inhibitor had no significant effect on the production of either PGF_{2α} (Fig. 3A) or PGE₂ (Fig. 3B).

Discussion

The present study reports coincident changes in the output of PGF_{2α}, PGE₂ and progesterone by human granulosa-lutein cells over the first 3 days of culture. The observation that declines in prostaglandin concentrations coincide with the rise in progesterone raises three possibilities. First, in view of the established inhibitory effect of PGF_{2α} on ovarian progesterone synthesis (reviewed in Richardson 1986, Auletta & Flint 1988, Michael *et al.* 1994, Olofsson & Leung 1994), the marked decline in PGF_{2α} production may facilitate the rise in progesterone synthesis by the luteinizing cells. Secondly, the increase in progesterone concentrations over the first 3 days in culture may suppress PGF_{2α} and PGE₂ output. Finally, there may be no causal relationship between the pattern of production of progesterone and the assayed prostaglandins as the cells luteinize *in vitro*. As discussed below, this report presents novel data which demonstrate that the decline in prostaglandin output and progressive rise in progesterone secretion from luteinizing human granulosa cells occur independently of each other and are not causally interdependent.

In order for endogenous synthesis of prostaglandins to determine progesterone production from luteinizing cells, the concentrations of those prostaglandins would need to approximate to the K_d values for the various subtypes of PGF_{2α} and PGE₂ receptors (FP and EP receptors respectively). On day 1 of culture this was indeed the case; the mean PGF_{2α} concentration of 14.0 ± 2.6 nM (i.e. 14.0 ± 2.6 pmol/10⁵ cells $\times$ 10⁵ cells/ml) approximates to the K_d of 13 nM for [³H]PGF_{2α} binding to the human CL (Rao *et al.* 1977) and exceeds the K_d for the cloned human FP receptor of 8.3 ± 2.5 nM (Lake *et al.* 1994). The situation for the receptor-mediated response to PGE₂ is complicated by the fact that, at present, there are four cloned subtypes of the EP receptor, designated EP1 to EP4, with K_d values that range from 1 nM for the human EP1 receptor to 11 nM for the human EP2 receptor (Coleman *et al.* 1994). Certainly, at the concentration of 4.0 ± 0.8 nM (measured on the first day of cell culture), PGE₂ produced by the luteinizing granulosa cells would be capable of binding to the EP2 and EP4 receptor subtypes which (through their ability to activate the cyclic adenosine-3',5'-monophosphate-protein kinase A pathway) have the capacity to stimulate ovarian steroidogenesis.

To test whether the progressive decline in PGF_{2α} output could account for the concomitant increase in progesterone production, cells were incubated with a range of concentrations of meclofenamic acid. This non-steroidal, anti-inflammatory agent inhibited prostaglandin output by up to $82 \pm 3\%$ when used in the high

nM to low μM concentration range, consistent with prior reports of the potency of meclofenamic acid as an inhibitor of PGHS activity in other cell types (Boctor *et al.* 1986, Zelinski-Wooten *et al.* 1990). However, in view of the novel observation that meclofenamic acid did not significantly affect progesterone output at concentrations of up to 10 μM , it seems unlikely that the endogenous production of prostaglandins in luteinizing human granulosa cells dictates the ability of such cells to synthesize progesterone.

In making the above observations, we note that the synthesis of $\text{PGF}_{2\alpha}$ appeared to be more sensitive to inhibition by meclofenamic acid than did the production of PGE_2 , even though the maximum degree of inhibition of both prostaglandins was comparable at the upper concentration of 10 μM meclofenamic acid. At concentrations of 0.01 and 0.1 μM , which are below the published IC_{50} for inhibition of cyclo-oxygenase/PGHS activity, meclofenamic acid significantly inhibited $\text{PGF}_{2\alpha}$ accumulation without significantly affecting PGE_2 synthesis. This discrepancy is hard to explain if one assumes that meclofenamic acid acts solely by inhibiting arachidonic acid metabolism at the level of the cyclo-oxygenase/lipoxygenase enzymes, and may indicate preferential inhibition of $\text{PGF}_{2\alpha}$ synthesis by low concentrations of meclofenamic acid. This could reflect direct effects of meclofenamic acid on PGF synthase activity and/or conversion of PGE_2 to $\text{PGF}_{2\alpha}$, catalysed by PGE_2 -9-ketoreductase. Alternatively, decreased intracellular concentrations of PGH_2 following inhibition of PGHS activity may have a greater impact on the generation of $\text{PGF}_{2\alpha}$ than PGE_2 due to the relative affinities of PGF and PGE_2 synthase for the decreased amount of prostaglandin substrate.

A consideration when evaluating changes in prostaglandin production is the potential of the cells of interest to oxidise prostaglandins to their 13,14-dihydro-15-keto metabolites. In the present study, the possible metabolism of $\text{PGF}_{2\alpha}$ and PGE_2 by 15-hydroxyprostaglandin dehydrogenase (PGDH) did not appear to be relevant since we have previously obtained two lines of evidence to indicate that human granulosa-lutein cells do not express significant PGDH activity, irrespective of the experimental treatments to which the cells have been exposed (A E Michael & D R E Abayasekara; unpublished data). First, while spent culture medium does contain 13,14-dihydro-5-keto- $\text{PGF}_{2\alpha}$ (PGFM) immunoreactivity, the apparent concentrations of PGFM in spent culture medium are typically less than 5% of the concentrations of $\text{PGF}_{2\alpha}$ and so can simply be attributed to cross-reaction of $\text{PGF}_{2\alpha}$ in the PGFM RIA. Moreover, when human granulosa-lutein cells are incubated with [^3H] $\text{PGF}_{2\alpha}$ for up to 24 h prior to the resolution of [^3H]-labelled metabolites by high performance liquid chromatography, we have been unable to identify any [^3H]PGFM. Hence, the suppression of $\text{PGF}_{2\alpha}$ and PGE_2 secretion from cells treated with meclofenamic acid and indomethacin reflects inhibition of

prostaglandin biosynthesis (at the level of PGHS) rather than stimulation of PG metabolism by PGDH.

To test whether the progressive rise in cellular progesterone output could account for the decline in $\text{PGF}_{2\alpha}$ and PGE_2 concentrations, cells were incubated with a range of concentrations of aminoglutethimide. This toxin has been used in a number of studies, including studies of human granulosa-lutein cells, to inhibit cytochrome P450 cholesterol side-chain cleavage and other cytochrome P450 members of the steroidogenic pathway. The concentration of aminoglutethimide required to significantly suppress progesterone secretion (100 μM) was consistent with that required to inhibit steroid biosynthesis in previous studies of ovarian and adrenal cells (Wilroy *et al.* 1968, Touitou *et al.* 1973, El-Hefnawy & Huhtaniemi 1998, Peluso & Pappalardo 1999). Despite decreasing progesterone production in the present study by $62 \pm 6\%$, 100 μM aminoglutethimide had no significant effect on the concentration of either prostaglandin measured. On the basis of this novel finding, we propose that the decline in $\text{PGF}_{2\alpha}$ and PGE_2 concentrations in spent medium as human granulosa cells luteinize *in vitro* is unlikely to be a consequence of the increasing progesterone production.

In experimental designs of the type employed in this study, it is usual to test whether the effects of the pharmacological inhibitors are specific and/or reversible by replacing the hormone that has been suppressed by the test compound. In those experiments that featured meclofenamic acid, it would have been usual to have treated the cells with PGE_2 to $\text{PGF}_{2\alpha}$, having suppressed endogenous prostaglandin synthesis, to establish whether any effects on progesterone production could be reversed. However, this element of the study was not justified since treatment of cells with meclofenamic acid had no significant effect on steroidogenesis in the first instance. Similarly, it was not necessary to treat cells exposed to 100 μM aminoglutethimide with exogenous progesterone since this cytochrome P450 inhibitor had no significant effect on concentrations of the measured prostaglandins.

The cell culture system used in this study was selected as a readily available functional approximation to the luteinizing granulosa cells of the newly formed CL. However, extrapolations between our *in vitro* observations and those biochemical changes that occur as human granulosa cells luteinize *in vivo* must be made with extreme caution for three major reasons. First, the granulosa cells employed in this and in similar studies have been exposed to pharmacological doses of gonadotrophins prior to isolation and culture. At this stage, it is not possible to comment on the precise biochemical impact of this endocrine history, but it would seem reasonable to assume, based on prior publications, that this might influence the *in vitro* output of both prostaglandins and steroid hormones. Secondly, the cell preparations obtained after centrifugation on 60% (v/v) Percoll contain a variety of cell types other than granulosa cells, of which most appear to

be ovarian macrophages, fibroblasts and erythrocytes. The contaminating erythrocytes are of limited significance since they do not adhere (and so are removed) during the course of cell culture, in addition to which they can be removed easily by gentle osmotic lysis. In our laboratories, we routinely assess the proportion of viable cells that are granulosa cells by assessing 3β -hydroxysteroid dehydrogenase (3β HSD) activity using an immunocytochemical test. When incubated with a 3β -hydroxy-, Δ^5 -androgen substrate (androst-5-ene- 3β ,17 β -diol), excess NAD^+ and nitroblue tetrazolium, $82 \pm 7\%$ (mean $\pm$ s.e. of eight independent measurements) of all viable cells stain positive for 3β HSD activity as reflected in the deposition of the insoluble reduced formazan salt. Hence we are confident that, in the present study, ovarian leukocytes plus fibroblasts would have accounted for less than 20% of the cultured cells. Clearly, any contaminating leukocytes might be expected to influence prostaglandin production from the granulosa cells through the local production of proinflammatory cytokines. However, we would argue that the newly forming human CL, which is a very highly vascularized gland, contains a large number of non-steroidogenic cells, particularly macrophages, which might similarly influence prostaglandin and progesterone output by luteinizing granulosa cells *in vivo*. The third and final factor that limits extrapolation between the data reported herein and the biochemical changes that occur as granulosa cells luteinize *in vivo* is our inability to accurately model the hormonal and cellular environment of the granulosa cells *in vitro*. In the newly forming CL, granulosa cells would be exposed to relatively high concentrations of luteinizing hormone (and, to a lesser extent, follicle-stimulating hormone) which we made no attempt to approximate since we had decided to examine changes in prostaglandin and progesterone output under true basal conditions. Moreover, in the immature CL, there appears to be a functional dialogue between the luteinizing granulosa and luteinizing theca cells which cannot be reproduced *in vitro* since it is not possible to isolate appreciable numbers of human theca cells from follicular aspirates. Taken together, these three major limitations on the interpretation of our data restrict the clinical significance of our data, but do not detract from the validity of our observations under the stated conditions.

Despite the caveats defined above, the model of cultured human granulosa-lutein cells is a widely used *in vitro* system which does appear to mirror many of the biochemical changes that occur when granulosa cells undergo functional luteinization *in vivo*. We are therefore confident that the biochemical changes reported herein are likely to occur as cells differentiate in the early luteal phase, subject to modulation by gonadotrophins, luteinizing theca cells, and paracrine agents produced by non-steroidogenic cells in the immature CL.

In summary, inhibition of endogenous prostaglandin synthesis by cultured human granulosa-lutein cells had no

significant effect on their progesterone output, and suppression of progesterone synthesis had no impact on the $\text{PGF}_{2\alpha}$ or PGE_2 concentrations in spent culture medium. We conclude, therefore, that the dynamic changes in the output of $\text{PGF}_{2\alpha}$, PGE_2 and progesterone from luteinizing human granulosa cells over the first 3 days of culture probably occur independently of each other.

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